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DISSEMINATED INTRAVASCULAR COAGULATION IN CRITICALLY ILL PATIENTS

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ACADEMIC DISSERTATION

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Do or do not. There is no try.

- *Yoda*

To Mika, Kalle and Vili

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals (I-IV). Articles have been reprinted with the kind permission of their copyright holders. In addition, some unpublished data are presented.

I Sivula M, Tallgren M, Pettilä V: Modified score for disseminated intravascular coagulation in the critically ill. *Intensive Care Med* 31:1209-1214, 2005.

II Sivula M, Pettilä V, Niemi TT, Varpula M, Kuitunen AH: Thromboelastometry in patients with severe sepsis and disseminated intravascular coagulation. *Blood Coagul Fibrinolysis* 20: 419-426, 2009.

III Sivula M, Hästbacka J, Kuitunen A, Lassila R, Tervahartiala T, Sorsa T, Pettilä V: Systemic matrix metalloproteinase-8 and tissue inhibitor of metalloproteinases-1 in severe sepsis-associated coagulopathy. *Acta Anaesthesiol Scand* 59:176-84, 2015.

IV Sivula M, Lakkisto P, Vaara S, Nisula S, Poukkanen M, Kuitunen A, Tikkanen I, Pettilä V, The FINNAKI study group: Histone-complexed DNA and high-mobility group box (HMGB) 1 in severe sepsis-associated thrombocytopenia and acute kidney injury: data from the prospective observational FINNAKI study. *Submitted.*

LIST OF ABBREVIATIONS

AKI	Acute kidney injury
AKIN	Acute Kidney Injury Network
APACHE	Acute Physiology and Chronic Health Evaluation
aPC	Activated protein C
aPTT	Activated partial thromboplastin time
AT	Antithrombin
AU	Absorbance unit
AUC	Area under curve
CFT	Clot formation time
CI	Confidence interval
CLP	Caecal ligation and puncture
CRP	C-reactive protein
CT	Clotting time
DIC	Disseminated intravascular coagulation
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
F1+2	Factor 1+2
FDP	Fibrin degradation product
FVa	Activated factor V
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXa	Activated factor X
GCS	Glasgow Coma Scale
hcDNA	Histone-complexed DNA
HMGB1	High-mobility group box 1
HPT	Haematopoietic malignancy
ICU	Intensive care unit
INR	International normalized ratio
ISTH	International Society on Thrombosis and Haemostasis
JAAM	Japanese Association of Acute Medicine
JMHW	Japanese Ministry of Health and Welfare
KDIGO	Kidney Disease: Improving Global Outcomes criteria
LI	Lysis index
LLN	Lower limit of normal
MA	Maximal amplitude
MCF	Maximal clot firmness
ML	Maximal lysis
MMP-8	Matrix metalloproteinase-8
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
NET	Neutrophil extracellular trap
OR	Odds ratio
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-activated receptor
PC	Protein C

POCT	Point-of-care test
PT	Prothrombin time
RAGE	Receptor for advanced glycation end products
RIFLE	Risk, Injury, Failure, Loss, and End-Stage criteria
ROC	Receiver operating characteristics
ROTEM	Rotational thromboelastometry
RRT	Renal replacement therapy
SAPS	Simplified Acute Physiology Score
SCr	Serum creatinine
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential Organ Failure Assessment
s-TM	Soluble thrombomodulin
t-PA	Tissue-type plasminogen activator
TAT	Thrombin-antithrombin complex
TEG	Thromboelastography
TEM	Thromboelastometry
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TG	Thrombin generation
TIMP-1	Tissue inhibitor of metalloproteinase-1
TLR	Toll-like receptor
TM	Thrombomodulin
TNF-α	Tumour necrosis factor alpha
u-PA	Urokinase-type plasminogen activator
u-PAR	Urokinase-type plasminogen activator receptor
ULN	Upper limit of normal
vWF	von Willebrand factor

ABSTRACT

Aims

Thrombocytopenia and the most severe form of coagulation disturbance, disseminated intravascular coagulation (DIC), are both frequent findings in critically ill patients and especially in those with severe sepsis. Patients with coagulation disturbance develop more organ dysfunction and have higher mortality. The pathophysiology behind the increased morbidity and mortality remains unclear.

The focus of this thesis was on assessing the applicability of diagnostic tools for DIC: a modification of a score suggested by the International Society on Thrombosis and Haemostasis (ISTH), and thromboelastometry (TEM), a whole-blood viscoelastic coagulation monitor. A further aim was to evaluate matrix metalloproteinase-8 (MMP-8), tissue inhibitor of metalloproteinase-1 (TIMP-1), nucleosomal histone-complexed DNA (hcDNA) and high-mobility group box 1 (HMGB1) protein levels in patients with and without DIC or thrombocytopenia and to assess their association with organ dysfunctions and outcome.

Patients and methods

Studies I-IV comprised 769 patients. Study I was a retrospective cohort study. All patients admitted to the multidisciplinary intensive care unit (ICU) of Helsinki University Hospital between 1.1.2002 and 31.10.2003 were screened for intensive care diagnoses. A modified score for overt DIC based on daily laboratory routines was calculated on days 1 to 7 or until discharge if that occurred earlier, and daily antithrombin was recorded, if available.

Study II was a prospective pilot study that included 28 patients with severe sepsis at admission. Diagnosis of overt DIC was based on daily calculations of modified DIC score. Ten healthy persons served as controls. TEM analyses using two different ROTEM® tests, EXTEM and FIBTEM, were performed on day 1 immediately after admission to ICU.

Study III was a prospective pilot study that contained 22 patients with severe sepsis at the time of admission or within 48 hours prior to admission. Serum MMP-8 and TIMP-1 concentrations were measured by time-resolved immunofluorometric and enzyme-linked immunosorbent assays (ELISA) at admission, and on days 2, 4 and 7, simultaneously with traditional coagulation assays and fibrin formation markers.

Study IV was a sub-study of the FINNAKI study, a large, prospective, observational, multicentre study conducted in 17 Finnish ICUs over a five-month period. Of the 918 patients with severe sepsis at admission or within the first 24 hours in the ICU, we

included a sub-cohort of 225 consecutive patients with admission plasma sample available for analyses. Admission levels of hcDNA and HMGB1 were analysed by commercial ELISA assays. The kinetic analysis comprised 49 patients for whom hcDNA and HMGB1 were measured at 0, 24 and 48 hours.

Main results

The incidence of DIC ranged from 31% in unselected ICU patients with appropriate underlying disease (I) to 41% (III) and 43% (II) in patients with severe sepsis. In Study IV, 33% of the patients developed thrombocytopenia, with platelet count <100 $x10^9$ /litre.

In DIC, 28-day mortality ranged from 40% to 44% (I-III); in patients with thrombocytopenia 90-day mortality was 39% (IV).

Study I demonstrated that of the components in the modified ISTH score, platelets, prothrombin time (PT) ratio and D-dimer either excellently or well, and fibrinogen only poorly discriminated the patients with overt DIC by receiver operating characteristics (ROC) curve analysis. Antithrombin possessed good discriminative power, comparable to PT ratio and D-dimer. DIC diagnosis based on the score was not an independent predictor of 28-day mortality.

In Study II, traditional coagulation tests showed a consistently worsening coagulopathy from healthy controls to patients without DIC and those with DIC. TEM analysis revealed that in patients with DIC clot formation time (CFT) was prolonged and maximal clot firmness (MCF) was decreased relative to both patients without DIC and healthy controls, indicating hypocoagulation. Patients without DIC had a similar TEM profile to controls, except that MCF showed a trend for hypercoagulation. In all patients, fibrinolysis was inhibited. EXTEM CFT, α-angle and MCF discriminated patients with DIC well.

Study III showed that MMP-8 and TIMP-1 concentrations were elevated in severe sepsis. MMP-8 was higher on day 2 in DIC patients than in patients without DIC. TIMP-1 was higher on days 1 and 2. TIMP-1 correlated negatively with platelet count, several coagulation parameters and disease severity scores.

In Study IV, hcDNA and HMGB1 levels were elevated in patients with thrombocytopenia or acute kidney injury (AKI) and in those who died within 90 days. HcDNA independently predicted thrombocytopenia, whereas HMGB1 was a predictor of the severest stage of AKI and of 90-day mortality.

Conclusions

In the university hospital multidisciplinary ICU, incidence of DIC was 31% in unselected patients with appropriate underlying diagnosis and over 40% in those

with severe sepsis. A prospective multicentre cohort study revealed a lower incidence (33%) of thrombocytopenia in severe sepsis patients. Components of the modified score for overt DIC discriminated patients with DIC well, except that fibrinogen proved useless. Discriminative power of AT was comparable to D-dimer and PT ratio. TEM analysis revealed that patients with DIC had hypocoagulable TEM trace as compared with sepsis patients without DIC. Clot strength and clot formation parameters discriminated DIC patients well from those without DIC. The markers speculated to contribute to coagulation disturbance and organ dysfunction, MMP-8, TIMP-1, hcDNA and HMGB1, were higher in patients with disturbed coagulation. HcDNA and HMGB1 were also elevated in patients with AKI and adverse outcome. HcDNA was an independent predictor of thrombocytopenia.

Keywords

Disseminated intravascular coagulation, severe sepsis, organ dysfunction, mortality, thrombocytopenia, thromboelastometry, matrix metalloproteinase-8, tissue inhibitor of metalloproteinase-1, nucleosomes, high-mobility group box 1

1. INTRODUCTION

Critically ill patients, regardless of their underlying disease, are prone to blood coagulation disorders. Several factors, including chronic illnesses, disturbed homeostasis, acquired platelet dysfunction, minor and major surgical procedures, immobilization, vascular catheters, extracorporeal circuits and medications, predispose to both bleeding and thrombotic complications.[1] In the intensive care unit (ICU), approximately 40% of the patients develop thrombocytopenia (defined as platelet count <150 $x10^9$ /litre),[2,3] and an international normalized ratio (INR) is increased in 30%.[4] Disturbed coagulation and its consequences may lead to organ dysfunction, prolonged hospitalization and increased mortality.

Severe sepsis is a common life-threatening condition in which an infectious agent triggers a series of proinflammatory reactions that manifest as haemodynamic imbalance, organ dysfunction and almost universal signs of coagulation abnormalities.[5] In addition to careful monitoring and customized medication, these patients are often administered excessive fluid therapy, ventilator support and renal replacement therapy (RRT) because of manifestations of organ dysfunction.

The most severe form of coagulation disturbance is disseminated intravascular coagulation (DIC), which may occur in several critical conditions, including inflammatory diseases, such as severe sepsis, trauma, organ destruction (e.g. pancreatitis) and obstetric emergencies. In DIC, intravascular coagulation is activated without macroscopic vessel injury, which leads to thrombocytopenia and consumption of coagulation factors. In addition, inactivation of the natural anticoagulant system and inhibition of fibrinolysis may occur, rendering the patients susceptible to both bleeding and thrombosis. Microvascular fibrin deposition and thrombosis occurring in DIC are thought to contribute to the development of multiple organ dysfunction and to increase mortality, although the mechanisms have been under debate.[6]

To standardize and facilitate the diagnosis of DIC, the International Society on Thrombosis and Haemostasis (ISTH) has proposed criteria and a score for overt DIC based on easily available global coagulation assays.[7] Several studies have proven the ISTH score applicable in early recognition of DIC, but milder coagulation disturbance still lacks proper diagnostic criteria, despite an apparent association with increased mortality.[8-10] From the clinical point of view, these patients are not prone to bleeding, contrary to what traditional coagulation assays may suggest. To date, no widely accepted treatment for severe sepsis-associated DIC exists.

The pathophysiology behind the development of sepsis-related organ dysfunction and failure is very complex. Suggested mechanisms include exaggerated interactions between numerous inflammatory mediators and regulators, cell-cell interaction molecules, cell apoptosis and excessive activation of coagulation.[11] Endothelial dysfunction seems to be a key step, however, little is known about the exact mechanisms or differences in susceptibility of organs.

The main focus of this thesis was to assess incidence of DIC with a score suggested by ISTH in ICU patients with appropriate underlying diseases known to be associated with DIC, and to evaluate blood coagulation in severe sepsis with a viscoelastic method, thromboelastometry. Another focus was on the pathophysiology of sepsis-related coagulation disturbance: separate studies investigated the roles of extracellular matrix-degrading proteins and their inhibitory enzyme, and circulating nucleus-derived proteins in the development of thrombocytopenia or DIC, and organ dysfunction.

2. REVIEW OF THE LITERATURE

2.1 HAEMOSTASIS IN VASCULAR DAMAGE

Haemostasis is a normal physiological process that causes blood to clot at a site of vascular injury. In primary haemostasis, activated platelets adhere to the site of damage and aggregate, forming a primary clot. This provokes a series of reactions, which finally form a tight fibrin network between aggregated platelets.

2.1.1 Platelets and primary haemostasis

Platelets are small anucleate cells that are fragments of the membrane, cytoplasmic organelles and granules of megakaryocytes and are composed in bone marrow. After release into the blood, platelets circulate for 7-10 days before being phagocytized by the spleen or the liver. Inactivated platelets circulate as discoid plates.[12]

The primary function of platelets is to maintain vascular integrity. When the vascular wall is injured for any reason, circulating platelets interact with exposed material, adhere to the site of injury and become activated. Upon activation, the cytoskeleton of the platelet rapidly rearranges, and platelets spread and form thin sheets (lamellae) and long thin processes (filopodia).[12] In this form, platelets start to form an initial plug to stop the bleeding and correct the damage.

Platelets interact with other cells, extracellular matrix and soluble compounds by several groups of membrane receptors. Interaction facilitates activation, adhesion and aggregation phases. Thrombin, a major mediator of the coagulation process, binds to protease-activated receptors (PARs), the main one being PAR-1. PAR-1 mediates platelet activation and amplifies the coagulation process.[13] At the site of injury, platelets adhere to exposed collagen by integrin-type receptors, especially glycoprotein (GP) Ib/V/IX, VI and Ia/IIa. Von Willebrand factor (vWF), on the surface of exposed collagen, serves as a major ligand for GP Ib. This receptor-vWF interaction reduces the velocity of platelets in blood vessels and allows them to attach to the site of injury. Platelet aggregation occurs mainly by the most abundant integrin, GP IIa/IIIb, which binds to fibrinogen, fibrin and other ligands, promoting aggregation of platelets.[13,14]

Activated platelets secrete numerous compounds from their α granules, dense granules, lysosomes and cytoplasmic stores, all contributing to platelet adhesion, aggregation and modulation of endothelial function and inflammatory processes. Nowadays, platelets are considered vital to host immunity.[15]

2.1.2 Coagulation process

Historically, blood coagulation has been seen as a sequential process in which coagulation factors first become activated and then activate the following factors in predefined order.[16,17] The waterfall cascade model included both intrinsic and extrinsic pathways, which were thought to unite at the level of factor X (FX) and form a final common pathway. The main reaction in the coagulation process is the formation of thrombin, which digests fibrinogen into fibrin, the main constituent of a blood clot.

After the introduction of the cascade model, it was soon realized that it could not explain all of the phenomena seen in patients with congenital coagulation factor deficiencies. Moreover, a well-functioning extrinsic pathway was unable to compensate defects on the intrinsic pathway and *vice versa*. Several factors were found to interact, irrespective of their order in the cascade.[18]

Nowadays, activation of coagulation is thought to occur mainly on cell surfaces, and cells control the process actively. Instead of a mechanical cascade model, coagulation occurs as overlapping phases on different cell surfaces.[19-21]

Initiation phase: Tissue factor (TF), a specific cell surface molecule, is exposed to circulating blood either by endothelial injury or inflammation. TF activates factor VII (FVII) and, as a consequence, FX becomes activated and produces thrombin in small amounts.

Amplification phase: Blood components, platelets, vWF and factor VIII (FVIII) come into contact with small amounts of thrombin on TF-bearing cells. Thrombin fully activates platelets as well as a number of coagulation factors, factors V, VII and XI.

Propagation phase: The reaction greatly amplifies. Factor IX combines with activated FVIII and activates FX to FXa, which combined with activated FV (FVa) converts large amounts of prothrombin to thrombin. As a result, fibrinogen is cleaved to fibrin monomers, which polymerize and in the presence of activated factor XIII form a stabilized fibrin mesh and a thrombus with initial platelet clot.

2.1.3 Regulation of coagulation

Natural anticoagulant mechanisms control fibrin formation tightly to keep the process localized. Tissue factor pathway inhibitor (TFPI) is the major regulator of the initiation phase, whereas antithrombin (AT) and activated protein C (aPC) inhibit thrombin formation in the propagation phase.[20] TFPI forms a complex with activated FVII (FVIIa) and FXa, thereby directly inhibiting the early phase of the coagulation process.[22] AT directly inhibits formed thrombin and other serine proteases.[23] Thrombin also binds to endothelial thrombomodulin, and this complex activates protein C. APC, with the contribution of protein S, cleaves factors VIIIa and Va and thereby inhibits propagation of coagulation.[24] In addition, platelets contain a large

variety of receptors and other signalling proteins that have a role in the regulation of thrombin formation and clot retraction.[25]

2.1.4 Fibrinolysis

Fibrinolysis is a sequence of enzymatic reactions, which start to degrade fibrin into soluble fibrin degradation products. The final aim is to remove the blood clot from the vessel wall as the epithelium heals, but fibrinolysis also regulates clot formation and keeps it localized.

Plasminogen is an inactive pro-enzyme that becomes activated to plasmin in the presence of fibrin by tissue- and urokinase-type plasminogen activators (t-PA and u-PA). Plasmin degrades fibrin network to degradation products, e.g. D-dimer, which can be measured.[26]

Several enzymes regulate fibrinolysis. Alpha-2-antiplasmin is the major inhibitor of plasmin. Plasminogen activator inhibitor-1 (PAI-1) inhibits both t-PA and u-PA, and it also plays a role in cell adhesion and migration.[27] Thrombin-activatable fibrinolysis inhibitor prevents binding of plasminogen to fibrin, thereby suppressing fibrinolysis.[28]

2.2 DISTURBED COAGULATION IN THE CRITICALLY ILL

Critically ill patients are susceptible to many coagulation disturbances. Patients may have multiple traumas and organ destructions, causing massive bleeding. Acute critical illness and many underlying diseases may activate the coagulation process without observable tissue injury. Many ICU-related factors, such as catheterizations, extracorporeal circuits, immobilization and certain drugs, predispose patients to either bleeding or thrombotic complications, or both. Often a patient may be prone to thrombosis and bleeding at the same time.[29,30]

2.2.1 Thrombocytopenia

Thrombocytopenia is a frequent finding in unselected critically ill patients. The definition of thrombocytopenia varies, but in general, platelet count below 150 $x10^9$ /l is considered mild, 50-99 $x10^9$ /l intermediate and <50 $x10^9$ /l severe thrombocytopenia. According to a recent systematic review, the prevalence of thrombocytopenia on admission to the ICU ranged greatly, from 8% to 68%, and the incidence during the course of ICU from 13% to 44% depending on the patient population.[31]

Thrombocytopenia is often multifactorial in origin. It may occur due to massive consumption (bleeding, disseminated intravascular coagulation or other thrombotic thrombocytopenic disorders), immune-mediated destruction, decreased production (toxic effects or viral infections), platelet sequestration to the spleen (liver and heart

diseases and haematological disorders), or a combination of these.[32,33] In the ICU setting, sepsis is one of the leading causes of thrombocytopenia, which occurs in over 50% of patients with septic shock.[34] Other causes are commonly used medications (piperacillin-tazobactam and other β-lactams, vancomycin, linezolid and heparin), especially after prolonged administration, and induced hypothermia.[35]

Thrombocytopenia predicts poor prognosis, is associated with bleeding complications and may delay important procedures. Several studies have shown that both thrombocytopenia *per se* and reduction in platelet count are independent predictors of death.[3,32,36,37] Patients with platelet count <50 $x10^9$ /l have a 3- to 5-fold risk of bleeding relative to those with a higher platelet count.[32,33,38,39]

2.2.2 Coagulation in severe sepsis

Severe sepsis is an overwhelming systemic inflammatory response to an infectious agent complicated by one or more acute organ failures. Septic shock is defined as hypotension refractory to adequate fluid resuscitation, and signs of insufficient perfusion.[40,41] Incidence of severe sepsis ranges from 0.48 to 3.0/1000/year.[42-45] Despite advancements in modern intensive care, mortality remains high, from 23% to >60% in those with at least four concomitant organ failures.[43,46,47]

Coagulation is activated in virtually all patients with a systemic inflammatory reaction. Inflammatory mediators, cytokines, chemokines and the complement system activate the endothelium and convert it into a prothrombotic surface. It has become clear that coagulation and inflammation are in tight crosstalk and strongly modulate each other.[11,48] **Figure 1** demonstrates a simplified representation of coagulation activation in inflammatory reactions.

1) **<u>Activation of coagulation</u>**

   In severe sepsis, TF has an essential role in initiation of coagulation. Cytokines, C-reactive protein and other inflammatory agents induce expression of TF on the surface of the endothelium and circulating monocytes, macrophages and microparticles, enucleated fragments from activated and apoptotic cells. This induction occurs in the presence of platelets and granulocytes and results in activation of the coagulation process, and finally, formation of thrombin.

2) **<u>Inhibition of natural anticoagulant mechanisms</u>**

   a. Proinflammatory cytokines may impair the attachment of TFPI on the endothelial surface, thereby reducing its function.

   b. AT activity is markedly reduced because of impaired synthesis, increased degradation and consumption due to binding to excessive amounts of thrombin.

c. The liver produces less PC. In addition, TM expression on the endothelium is down-regulated, and endothelium-bound TM is cleaved and released to the circulation. Soluble TM is much less active than endothelium-bound TM. As PC activation normally requires the presence of TM-bound thrombin, endothelial protein C receptor (EPCR) and co-factor protein S, these alterations inevitably reduce PC activation and anticoagulant capacity.

3) **Supressed fibrinolysis**

In sepsis, fibrinolysis-inhibiting enzyme PAI-1 levels increase in response to circulating tumour necrosis factor alpha (TNF-α) and interleukin-1β.[49] As a consequence, inadequate fibrin removal may lead to microvascular thrombosis.

4) **Coagulation-inflammation interaction**

Coagulation modulates inflammation by several mechanisms. PARs are receptors located on the surface of endothelium, monocytes, platelets and fibroblasts. Thrombin, TF/FVIIa-complex and FXa can activate PARs to produce inflammatory cytokines and growth factors.[50]

Activated platelets strongly contribute to host immunity by secreting and releasing many inflammatory mediators and interacting with most leukocytes. Activated and aggregated platelets may capture neutrophils and bring them in close contact with the disrupted endothelium.[15,51]

AT possesses potent anti-inflammatory properties. AT induces prostacyclin release, which, in turn, inhibits platelet activation and aggregation and decreases production of various proinflammatory agents. AT also directly blocks the interaction of leukocytes with endothelial cells.[48]

Anti-inflammatory effects of aPC are mainly mediated by EPCR. APC inhibits production of proinflammatory cytokines, inhibits leukocyte chemotaxis and adhesion, protects against disruption of endothelium and prevents endothelial apoptosis.[52]

Many fibrinolytic factors, in particular u-PA and its receptor u-PAR, mediate leukocyte adhesion and migration.[53] Potential mediators in this process are extracellular matrix-degrading proteases (plasmin and metalloproteinases), which are activated by u-PA and u-PAR.[54] PAI-1, instead, may inhibit this process.[55]

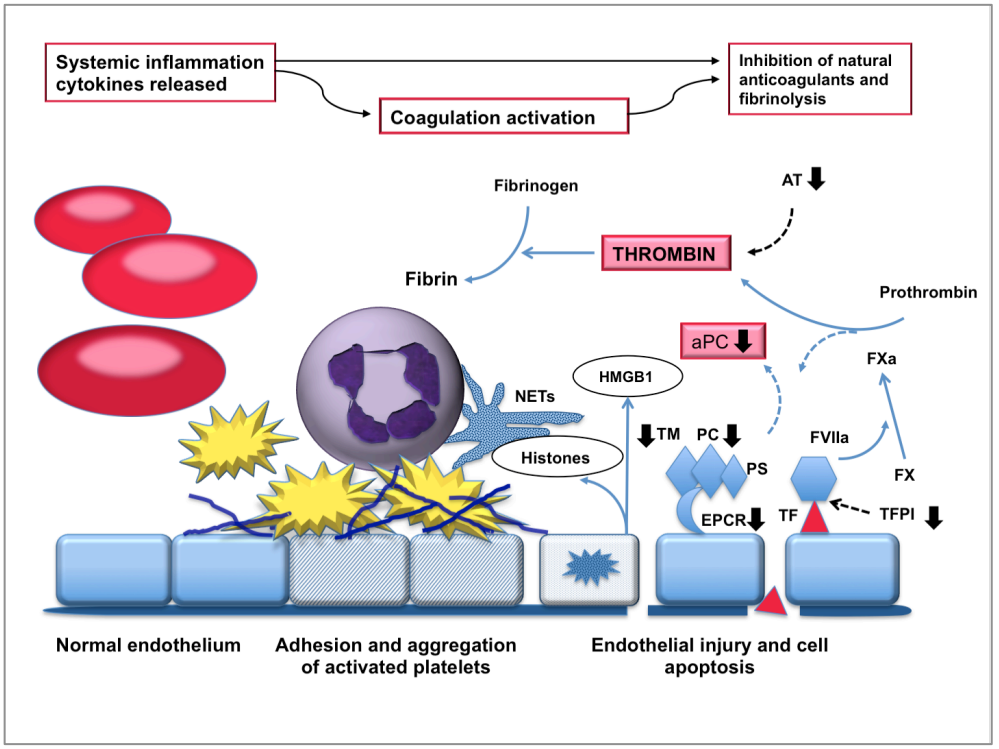


Figure 1. Simplified presentation of coagulation disturbance in severe sepsis.

In severe systemic inflammatory reaction, a large number of mediators, including cytokines, chemokines and components of the complement system, initiate an exaggerated activation of platelets and the coagulation system and the simultaneous inhibition of fibrinolysis. These compounds further activate the endothelium, which starts to express tissue factor (TF, red triangle). TF is also released to the circulation from other tissues by disruption of endothelial integrity. TF initiates series of enzymatic reactions, which lead to a thrombin 'burst'. The end-product of the coagulation process, fibrin, forms clots with activated and aggregated platelets on the surface of the activated endothelium. Apoptotic endothelial cells release intranuclear compounds, e.g. histones and high-mobility group box 1 (HMGB1) protein, which enhance both inflammation and coagulation reactions either directly or by neutrophil extracellular traps (NETs). Reduced amount of natural anticoagulants cannot suppress these reactions, and microvascular thrombosis may occur.

AT, antithrombin; aPC, activated protein C; EPCR, endothelial protein C receptor; FVIIa, activated FVII; FX, factor X; FXa, activated factor X; PC, protein C; PS, protein S; TM, thrombomodulin; TFPI, tissue factor pathway inhibitor.

2.2.3 Disseminated intravascular coagulation (DIC)

ISTH has defined DIC as 'an acquired syndrome characterized by the intravascular activation of coagulation with loss of localization arising from different causes. It can originate from and cause damage to the microvasculature, which if sufficiently severe, can produce organ dysfunction'.[7] The term 'overt DIC' refers to uncompensated coagulation, i.e. a severe form of the coagulation disorder. 'Non-overt DIC' is a milder coagulation disturbance with sufficient compensatory mechanisms.

DIC is a syndrome triggered by a variety of conditions: infectious and inflammatory diseases, malignancies, severe organ destruction, vascular anomalies, obstetric emergencies and immunological and toxicological emergencies.[56] Exaggerated global activation of coagulation occurs as a response to a systemic inflammatory reaction and/or a release of procoagulant material into the bloodstream. Briefly, in DIC, activation of microvascular endothelium and exposure of TF to circulating FVII leads rapidly to an overwhelming thrombin burst and formation of excessive amounts of fibrin, which malfunctioning natural anticoagulant and fibrinolytic systems cannot suppress.[6,57]

The incidence of DIC depends on the underlying disease. In severe sepsis, DIC exists in about 30-40% of patients.[58-60] In severe trauma, the incidence of overt DIC is somewhat lower, about 10% in the first 24 hours after trauma.[61] The presence of DIC may roughly double the mortality in critically ill patients to approximately 40%.[59,62-64] Based on the pathophysiology of DIC, fibrin deposition and subsequent microthrombosis may obstruct microvasculature, thus contributing to the development of multiple organ dysfunction.[65] Many studies show that the incidence and severity of DIC are directly correlated to the degree of organ dysfunction.[62,64,66]

Major bleeding due to thrombocytopenia and low levels of coagulation factors is the most feared consequence. However, it seems to be rather infrequent. Subgroup analyses of DIC patients receiving placebo in large anticoagulant trials have revealed that incidence of any bleeding was approximately 11% and major bleeding occurred in only 3%.[58,59] In an unselected cohort of critically ill patients, thrombocytopenia of any cause was associated with major bleeding in 20%.[67]

2.3 DIAGNOSTICS OF COAGULOPATHY

'Traditional coagulation assays' refer to easily accessible tests of blood coagulation capacity that measure the deficiency or consumption of the components. These tests include platelet count, prothrombin time (PT) with modifications, activated partial thromboplastin time (aPTT) and fibrinogen concentration. 'Fibrin-related marker' assays measure the extent of coagulation activation and fibrin formation.

Measuring platelet count is the most performed test for primary evaluation of the haemostatic system. The blood sample is anticoagulated, and counting is performed by either impedance, optical or immunological methods. Low platelet count can be verified manually to exclude agglutination.[68]

PT assay is a coagulation screening test used for assessing liver function, recognizing deficiencies or consumption of certain coagulation factors and monitoring oral anticoagulant therapy. It was first described by Quick[69] in 1935, and a later modification by Owren[70] is widely used in Nordic countries. Both Quick- and Owren-type PT assays measure vitamin K-dependent coagulation factors (FVII, FX and prothrombin), but Quick-type PT is dependent on FV and fibrinogen as well. Owren-type PT is often reported as a percentage of activity of studied plasma relative to a commercial calibrator representing 100% activity. Often PT is reported as an international normalized ratio (INR).[68]

APTT is a screening test for the deficiency of coagulation factors involved in 'intrinsic' and common pathways (factors V, X, II, VIII, IX, XI and XII and fibrinogen). However, prolonged APTT does not necessarily indicate increased risk of bleeding.[71]

D-dimer fragment is the terminal product of plasmin-induced degradation of cross-linked fibrin, and it is derived from both intravascular and extravascular clots. In plasma, D-dimer forms a complex with other compounds. Different monoclonal D-dimer assays recognize a mixture of D-dimer-containing complexes with variable sensitivity. Thus, numerical results from available commercial assays may vary widely.[72] These fibrin *degradation* tests report on the active coagulation process and clot formation only indirectly.

The main indication for fibrinogen assessment is active bleeding. In most cases, low levels indicate consumption of fibrinogen by blood loss or activated coagulation process. Fibrinogen concentration is measured in clinical settings by functional assays using, for example, the kinetic method of Clauss.[73]

2.3.1 Different coagulation tests and their combinations in DIC

Although pathophysiology of DIC has been extensively investigated, diagnosis of DIC on the basis of laboratory findings may be difficult. Combining and repeating the assays may increase specificity because none of the single routine assays alone is specific to DIC. A summary of the coagulation assays proposed in the diagnosis of DIC is presented in **Table 1.**

Table 1. Laboratory tests in the diagnosis of DIC.

Test	Pathologic results in all ICU patients	Pathologic results in DIC	Specific comments
Consumption of components			
Platelet count	<150 x10^9 /l (LLN): **35-44%** of medical ICU patients[3,32]	<150 x10^9/l (LLN): **>95%** <100 x10^9: **50-60%** <50 x10^9: **10-15%**[74]	Hallmark of DIC diagnosis: low or declining platelet count. Thrombocytopenia strong independent predictor of ICU mortality regardless of origin.[3]
Prothrombin time	INR >1.5: **30%** of ICU patients[4] PT>14.5 s (ULN): **93%** of patients with severe sepsis[59,75]	**>95%**[74,76]	Not very sensitive to early (milder) consumption (pathologic result occurs when concentration of any coagulation factor concerned is below 50%).[74] PT is prolonged (Owren-type PT ratio reduced) in later stages of DIC.[77]
Activated partial thromboplastin time	>39 s (ULN): **63%** of patients with severe sepsis[75]	**>95%**[59]	Because of elevated FVIII and fibrinogen due to acute phase reaction, aPTT may be even shortened in the early phase of DIC.[77]
Fibrin-related markers			
Fibrinogen		<1 g/l: **24%** >2 g/l: **69%** [78]	Acute phase protein. May be normal or elevated in early DIC.[77] Elevated levels correlate with mortality and organ failure.
D-dimer	>0.4 (ULN): **100%** of patients with severe sepsis[75]	**100%**[59]	High D-Dimer: low specificity. Normal D-dimer effectively rules out DIC. Problematic standardization due to several different assays.[74,77]
Coagulation inhibition			
Antithrombin	<80% (LLN): **82%** of patients with severe sepsis[75]	**>95%**[59]	Potentially useful, but low specificity. Shown to be associated with mortality.[23] Not globally available.[74,77]
Protein C	<81% (LLN): **87%** of patients with severe sepsis[75]	**>95%**[59]	Not globally available.[77]
Soluble thrombomodulin	>53 ng/ml (ULN): **72%** of patients with severe sepsis[75]	**>95%**[59]	Available only in specific coagulation laboratories.

Table 1 (continued). Laboratory tests in the diagnosis of DIC.

Test	Pathologic results in other conditions	Pathologic results in DIC	Specific comments
Fibrinolysis			
Plasminogen activator inhibitor-1	>37.8 AU/ml (ULN): **44%** of patients with severe sepsis[75]	**>95%**[59]	Potentially useful, but available only in specific coagulation laboratories.[74,77]
Other			
Fragment 1+2	>1.1 nmol/l (ULN): **78%** of patients with severe sepsis[75]	**>95%**[59]	Not specific to DIC. Pre-analytic factors may strongly affect the results. Available only in specific coagulation laboratories.[74]
Thrombin-antithrombin complex	>4.1 μg/l (ULN): **96%** of patients with severe sepsis[75]	**>95%**[59]	Not specific to DIC. Pre-analytic factors may strongly affect the results. Available only in specific coagulation laboratories.[74]

DIC, disseminated intravascular coagulation; ICU, intensive care unit; LLN, lower limit of normal; ULN, upper limit of normal; PT, prothrombin time; aPTT, activated partial thromboplastin time; AU, absorbance unit.

2.3.2 DIC score and its evolution

Previously, the diagnosis of DIC was based mainly on "expert opinion", local guidelines and pathological changes in coagulation tests because a universal definition was lacking.[79-84] In 2001, ISTH presented diagnostic criteria based on easily achievable 'global' coagulation assays.[7] A prerequisite for the use of the score is an appropriate underlying diagnosis that may cause DIC. A combination of parameters (platelet count, PT in seconds, fibrin-related marker and fibrinogen) gives points according to their level. Diagnosis of overt DIC is justified if the total score is at least 5. Since 2001, many study groups have modified the ISTH score by varying laboratory tests or their cut-off values. The role of fibrinogen in the score has been debated because of its dual behaviour in critical illness.[59,78,85]

In order to facilitate diagnosis of DIC, ISTH also suggested a template for a scoring system for stressed, but still compensated coagulation, non-overt DIC.[7] The template takes into account both absolute values of coagulation tests and their changes. Unlike the score for overt DIC, the score for non-overt DIC includes specific markers like AT and PC.[8,9,86]

As compared with an 'expert opinion', ISTH score recognizes overt DIC patients excellently and correlates well with degree of organ dysfunction and 28-day

mortality.[64] Several studies have assessed ISTH score against two other scoring systems: the Japanese Ministry of Health and Welfare (JMHW)[87] and the Japanese Association for Acute Medicine (JAAM)[88] scores. JAAM criteria seem to be most sensitive in the early diagnosis of DIC.[60] JMHW and JAAM scores have high sensitivity for DIC of any aetiology, whereas ISTH score is the most specific, but may miss milder cases of DIC and even non-survivors.[62] **Table 2** summarizes different scores for DIC.

No gold standard for the diagnosis of DIC exists. Recently, ISTH Scientific and Standardization Committee on DIC published a communication as an attempt to harmonize the different guidelines for DIC.[89-92] The committee suggested the use of any score without setting any preferences.

Table 2. Scoring systems for overt DIC.

Points:		0	1	2	3
Platelet count ($x10^9$/l)	**ISTH** JMHW JAAM	**≥100** >120 all with HPT ≥120	**50-99** 80-120 80-120 or >30%fall/ 24h	**<50** ≤80 -	- ≤50 <80 or >50%fall/ 24h
Prolongation of PT (seconds or ratio)	**ISTH** JMHW JAAM	**<ULN + 3s** <1.25 <1.2	**ULN + 3-6s** ≥1.25 ≥1.2	**>ULN + 6s** ≥1.67 -	
Elevated fibrin formation/ degradation related marker	**ISTH** JMHW JAAM	**No increase** FDP: <10 FDP: <10	**-** FDP: ≥10 FDP: 10-25	**Moderate** FDP: ≥20 -	**Strong** FDP: ≥40 FDP: ≥25
Fibrinogen (g/l)	**ISTH** JMHW JAAM	**≥1.0** ≥1.5 -	**<1.0** ≤1.5 -		
Additional points	**ISTH** JMHW JAAM	**None** Underlying disease, organ failure due to thrombosis: +1 point, bleeding symptoms in non-HPT patients: +1 point, ≥3 SIRS criteria: +1 point		**DIC diagnosis** **ISTH: ≥5** JMHW: ≥7/ ≥4 (HPT-/+) JAAM: ≥4	

ISTH, International Society on Thrombosis and Haemostasis; JMHW, Japanese Ministry of Health and Welfare; JAAM, Japanese Association of Acute Medicine; HPT, haematopoietic malignancy; PT, prothrombin time; FDP, fibrin degradation products; ULN, upper limit of normal; LLN, lower limit of normal; SIRS, systemic inflammatory response syndrome.

2.4 POINT-OF-CARE HAEMOSTASIS TESTS

Point-of-care test (POCT) refers to a diagnostic test that can be performed near the patient, and the results are readily available, improving patient care in rapidly changing situations.[93] As POCTs are frequently performed by personnel without training for laboratory analytics, the procedure must be well controlled. The British Committee for Standards in Haematology has published guidelines to standardize conditions for POCT testing.[94]

Although severe sepsis-related coagulopathy may not demand immediate actions unlike acute haemorrhage, POCT analyses, including thromboelastometry/ -graphy (TEM/TEG) may provide additional information on coagulation by visualizing clot formation as a continuous process.[95] The ISTH Scientific and Standardization Committee on DIC has encouraged the study of applicability of POCTs in DIC.

2.4.1 Methodology of rotational thromboelastometry

Hartert[96] introduced a classical TEG for research purposes in 1948. It took, however, several decades until TEG gained a foothold in clinical practice, first as a method to assess coagulation and decrease blood loss in liver transplantations and cardiac surgery.[97,98] Development of the method over the years has led to different technical solutions. The main principle of different commercial devices is the same; the methodology produces bed-side a real-time graphical trace of visco-elastic forces during clot formation, giving information on initiation of coagulation, fibrin polymerization, clot strengthening and fibrinolysis. TEG summarizes the effect of coagulation factors, platelets, anticoagulants and fibrinolytic factors.

Rotational TEM, ROTEM® (Tem International GmbH, Munich, Germany), is an application developed on the basis of the original TEG. Citrated blood sample is first pipetted into a plastic cuvette located in a prewarmed (37°C) cup holder. After adding necessary reagents to blood, an oscillating pin starts to rotate and form a clot between the cup and the pin (**Figure 2**). A graphical curve presents an optically detected change in viscosity of the sample (**Figure 3**).

TEM tests assess different aspects of coagulation. The most common tests are:

1. **EXTEM:** assesses the combined effect of extrinsic pathway coagulation factors and platelets. Citrated blood is recalcified after which TF-containing reagent activates coagulation. EXTEM is not affected by heparin.

2. **FIBTEM:** In recalcified blood, addition of TF triggers coagulation and cytochalasin D eliminates the effect of platelets. FIBTEM assesses firmness of a fibrin clot, and trace is mainly influenced by fibrinogen and factor XIII levels.

3. **HEPTEM:** Activation of coagulation occurs similarly to INTEM assay, but heparinase-containing reagent eliminates the effect of heparin. Comparison with INTEM reveals the effect of heparin.

4. **APTEM:** In APTEM, addition of aprotinin inactivates plasmin immediately and prevents fibrinolysis. In the case of suspected hyperfibrinolysis, APTEM and EXTEM are compared.

5. **NATEM:** Recalcified sample is allowed to coagulate without any activators in this original, non-activated TEM test. Not in clinical use.

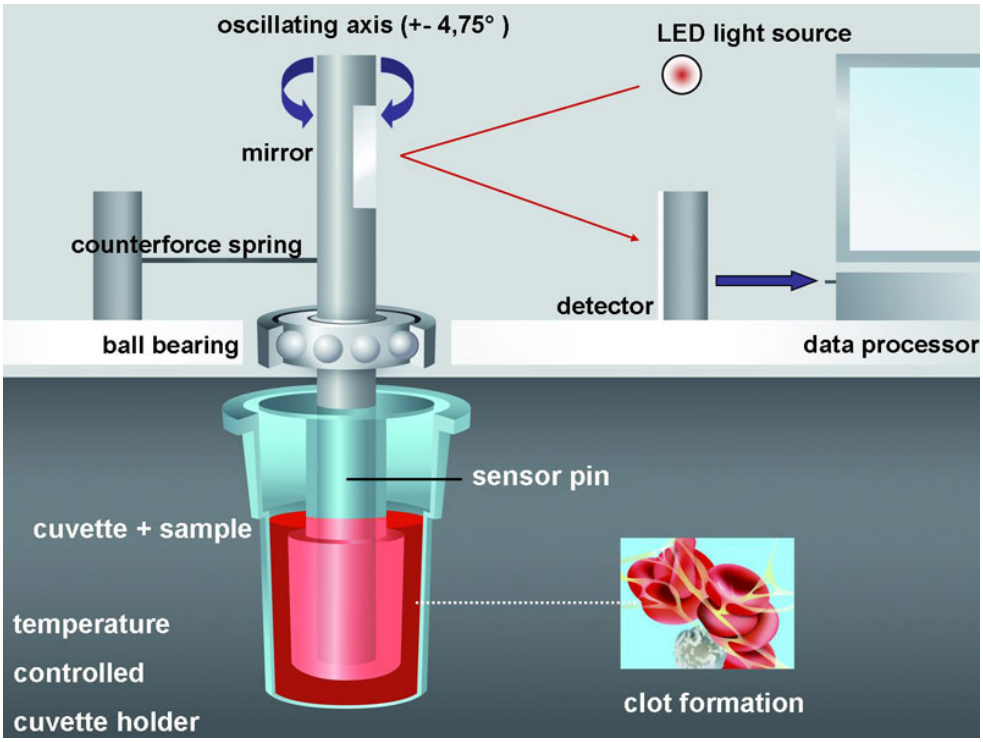


Figure 2. Detection method of TEM.

Figure published with the permission of Tem International GmbH.

The following parameters can be derived from the graphic curve:

1. **Clotting time (CT):** A period from the addition of the activating reagents until the recognizable trace of fibrin formation (amplitude of 2 mm) reflects initial fibrin formation.

2. **Clot formation time (CFT):** A period from the first signs of clot formation until the clot formation trace reaches an amplitude of 20 mm. CFT reflects fibrin build-up and clot formation kinetics.

3. **Alpha angle (α):** An angle between the baseline and the tangent of the trace at the point where the amplitude reaches 2 mm. α reflects the speed of clot formation.

4. **Maximal clot firmness (MCF):** The maximal amplitude of thrombo-elastometry trace reflects the strength of a clot and the combined effect of coagulation factors and platelets.

5. **Lysis index (LI):** Percentage of amplitude at given time point relative to MCF reflects the speed of fibrinolysis.

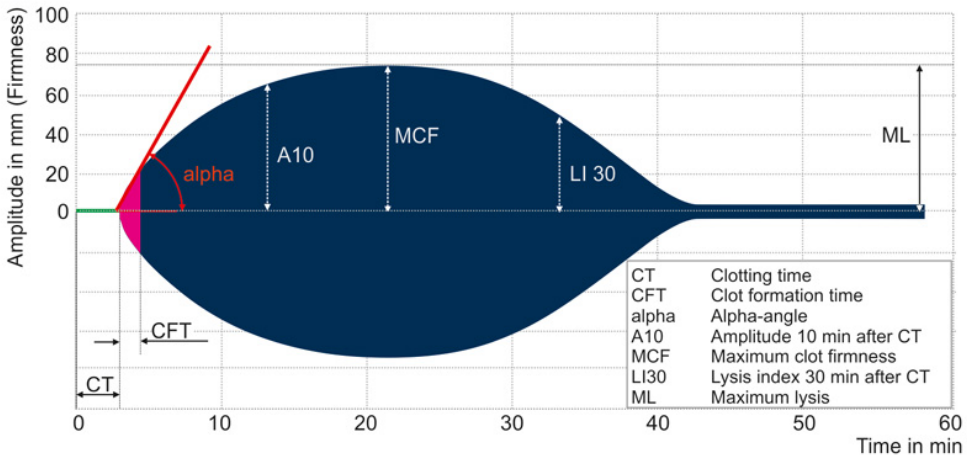


Figure 3. TEM curve with parameters.

Figure published with the permission of Tem International GmbH.

2.4.2 Thromboelastometry/ -graphy in septic coagulopathy

TEM/TEG has slowly gained popularity in acute and intensive care settings. The interest has been greatest among professionals who take care of trauma patients with rapid and marked changes in coagulation capacity.[99-101] In unselected cohort of critically ill patients, hypocoagulation diagnosed by TEG predicted independently 30-day mortality.[102]

The few endotoxemia model studies have demonstrated quite consistently that activation of coagulation occurs more rapidly, but clot formation and clot strength are either reduced or not affected depending on the dose of lipopolysaccharide.[103-105] In studies on septic patients TEM/TEG findings vary widely depending on the population, time course of the disease, chosen technology and the tests applied. Although at least minor coagulation activation occurs in virtually every patient with severe sepsis, only more marked disturbance is detectable with TEM/TEG. Thus, most studies examining severe sepsis patients as a homogeneous group have shown that TEM/TEG parameters either remain within reference ranges or demonstrate mild hypercoagulability.[106-113] However, patients with more severe condition (SOFA score >10 or overt DIC) are hypocoagulable according to TEM, which is associated with a higher mortality.[114,115] Some studies have reported inhibition of fibrinolysis in otherwise normocoagulable patients.[109-112,116] **Tables 3 and 4** summarize the greatly varying study designs and the main results of experimental and clinical TEM/TEG studies.

A recent systematic review assessed applicability of TEM/TEG in detecting sepsis-related coagulation disorders and in predicting outcome.[117] The authors conclude that hypocoagulability as detected by TEM/TEG may aid in diagnosing DIC and predicting mortality, but diagnostic accuracy of TEM/TEG in general sepsis-related coagulopathy is limited because of the dynamic nature of the coagulation process.

Table 3. Endotoxemia studies on rotational TEM.

Study	Objects	TEM tests	Design	Results
Spiel 2005 [103]	22 healthy males 4 controls	ROTEM: NATEM	Endotoxemia induced by bolus of LPS (2 ng/kg) Blood samples at 0, 1, 2, 3, 4, 6, 8 and 24 hours	**CT:** ↓ until 6 hours, after which changes diminished. **CFT:** no change **MCF:** no change **ML:** 3.9-fold ↑ at 2 hours **Traditional tests:** Platelets slightly ↓
Velik-Salchner 2009 [104]	15 pigs	ROTEM: INTEM, EXTEM and FIBTEM	Endotoxemia induced by a bolus and infusion of LPS (>200 μg/kg). Blood samples at 0 and 60 minutes.	**CT:** ↓ in INTEM, but was unchanged in EXTEM **CFT:** ↑ both in EXTEM and in INTEM **MCF:** ↓ in all tests **Traditional tests:** PT, aPTT, D-dimer: no change Platelets, fibrinogen, Antithrombin ↓
Schöchl 2011 [105]	10 pigs	ROTEM: NATEM, FIBTEM	Endotoxemia induced by LPS infusion (ad 10 μg/kg). Blood samples at 0, 1, 2, 3, 4 and 5 hours	**CT:** ↓ until 3 hours, after which changes diminished. **CFT:** ↑ until 5hours **MCF:** ↓until 5 hours

TEM, thromboelastometry; LPS, lipopolysaccharide; CT, clotting time; CFT, clot formation time; MCF, maximal clot firmness; ML, maximal lysis; PT, prothrombin time, aPTT, activated partial thromboplastin time.

Table 4. Clinical studies on TEM/TEG in human sepsis.

Study	Patients	TEM/TEG	Design	Results
Collins 2006 [106]	n= 38 severe sepsis n= 32 healthy controls	ROTEM®: EXTEM	Prospective Blood sampling time: not reported	• CT ↑, MCF and α ↑: activation of clot formation delayed, but once initiated, then normal or exaggerated
Gonano 2006 [107]	Substudy of Kybersept Trial n=16 (placebo) n=17 (AT) no controls	TEG®: Heparinase-TEG, Abciximab -TEG	Blood samples prior AT and then daily	• Reaction time: ↓ Coagulation time: ↓, Maximal amplitude: ↑ • Hypercoagulation relative to reference values
Daudel 2009 [108]	n=30 severe sepsis or septic shock	ROTEM®: INTEM, EXTEM, HEPTEM, FIBTEM	Prospective cohort study Blood samples at 0, 12, 24 and 48 hours	• Results within reference values • SOFA>10 vs. <10: MCF ↓, CFT ↑, α: ↓, CT: no change
Sharma 2010 [118]	n=21 overt DIC n=21 no overt DIC	TEM-A® non-activated test	Prospective Blood sampling time: not reported	• TEM score for DIC: 1 point for each hypocoagulable TEM parameter. • TEM score ≥2: ROC AUC 0.957 (0.902-1.0), sensitivity 95%, specificity 81%.
Adamzik 2010 [109]	n=56 severe sepsis n=52 postoperative n=NA controls	ROTEM®: NATEM with heparinase	Observational cohort study Blood samples within 24 hours after diagnosis/ operation	• **Sepsis vs. controls:** CT ↓, CFT↓, α ↑; MCF no change • **Postoperative:** hypercoagulability • LI: ↑ sepsis vs. postoperative and controls → inhibition of fibrinolysis. • LI ROC AUC 0.901, OR 85.3 for severe sepsis

Table 4 (continued). Clinical studies on TEM/TEG in human sepsis.

Study	Patients	TEM/TEG	Design	Results
Adamzik 2011 [114]	n=98 patients with severe sepsis	ROTEM®: NATEM with heparinase	Cohort study Blood samples on admission	• In non-survivors: CT unchanged; CFT↑; α↓; MCF↓ • Changes in CFT, α, MCF predict 30-day mortality better than SAPS II or SOFA
Brenner 2012 [110]	n=30 septic shock n=30 surgical n=30 controls	ROTEM®: INTEM, EXTEM, HEPTEM, FIBTEM	Blood samples on days 1,2, 4,7,14 and 28	• Sepsis patients (whole cohort) normocoagulable except • MCF ↑ (FIBTEM), CT ↑ (INTEM) and LIs ↑ (EXTEM) • Non-DIC sepsis patients hypercoagulable • DIC patients hypocoagulable
Durila 2012 [111]	n=38 surgical oesophagec-tomy	TEG®: Native TEG	Blood samples prior to the operation and once daily until day 6	• Reduced clot lysis in septic patients
Massion 2012 [112]	n=39 septic shock	ROTEM®: INTEM, HEPTEM, FIBTEM	Blood samples at 0 and 6 hours, and on days 1, 2, 3 and 7	• Normocoagulability by TEM • Hypocoagulability by traditional and thrombin generation assays • Hypofibrinolysis: LI60 higher • Prediction of hospital mortality: aPTT, TG

Table 4 (continued). Clinical studies on TEM/TEG in human sepsis.

Study	Patients	TEM/TEG	Design	Results
Ostrowski 2013 [115]	n=50 severe sepsis or septic shock	TEG®	Blood samples on days 1,2,3 and 4	• 22%/ 48% / 30% hypo- /normo- /hypercoagulable • TEG constant until Day 4 • Hypocoagulable MA predicted 28-day mortality • Reduced platelet contribution to MA in the hypocoagulable
Andersen 2014 [113]	n=36 severe sepsis or septic shock	ROTEM®: INTEM, EXTEM, HEPTEM, FIBTEM	Blood samples on days 1,2,3 and 7	• Normocoagulability in ROTEM, mild hypocoagulation in PT, aPTT and platelet count. • Trend for mild hypocoagulation in DIC patients.
Haase 2015 [119]	n=260 severe sepsis	TEG®: native and fibrinogen tests	Samples at admission, and daily until day 5	• TEG stable for 5 days • Fibrinogen MA ↑ • Most patients: clotting time ↑, MA near ULN • Hypocoagulation correlated with SOFA; independent risk factor for mortality
Prakash 2015[116]	n=77 sepsis	ROTEM®: NATEM PAI-1	Samples at admission, and daily until day 3	• Inhibition of fibrinolysis correlated with a degree of organ dysfunctions (ML↓, PAI-1↑)

aPTT, activated partial thromboplastin time; AT, antithrombin; AUC, area under curve; CT, clotting time; CFT, clot formation time; DIC, disseminated intravascular coagulation; LI, lysis index; MA, maximal amplitude; MCF, maximal clot firmness; ML, maximal lysis; OR, odds ratio, PT, prothrombin time, ROC, receiver operating characteristics; SAPS, Simplified Acute Physiology Score; SOFA, Sequential Organ Failure Assessment; TG, thrombin generation; TEG, thromboelastography; TEM, thromboelastometry; ULN, upper limit of normal.

2.5 ORGAN DYSFUNCTION

2.5.1 Sepsis and multiple organ dysfunction

'Sepsis' refers to a host response to a suspected or confirmed infection, manifested by at least two of the four criteria of systemic inflammatory response syndrome (SIRS). Sepsis is graded as 'severe' when hypoperfusion, hypotension or any organ dysfunction coexists. In 'septic shock', sepsis-induced hypotension despite adequate fluid resuscitation occurs, and signs of hypoperfusion exist. Severe sepsis may lead to multiple organ dysfunction syndrome (MODS) in which function of more than one organ is defective and the organs are unable to maintain homeostasis.[40,41] MODS accounts for the high mortality seen in the critically ill.[120]

According to a recent review, the hallmark of MODS is a hyperinflammatory response to a triggering incident. First, exaggerated production of proinflammatory mediators results in universal dysfunction of endothelial cells and adhesion of leukocytes to the activated endothelium. Secondary mediators and reactive oxygen species amplify this reaction. Later, intrinsic inflammatory cells in the organs sustain the inflammation.[121] Different organs respond to inflammatory mediators in typical ways, others being more vulnerable to the development of dysfunction.[122-124]

2.5.2 Acute kidney injury

Acute kidney injury (AKI) is defined as a 'common syndrome with an abrupt decrease in kidney function, which includes, but is not limited to, acute renal failure. AKI may arise from various aetiologies, including pre-renal causes, acute specific kidney processes and post-renal obstructive nephropathy'.[125] Manifestation of AKI forms a continuum from minor biomarker changes to full-blown acute failure of kidney function requiring RRT.

To rein in multiple and conflicting definitions for AKI, three subsequent, widely acknowledged criteria for AKI have been published since 2004: the Risk, Injury, Failure, Loss and End-stage renal disease (RIFLE) classification,[126] the AKI Network criteria (AKIN)[127] and, last, the Kidney Disease: Improving Global Outcomes (KDIGO) criteria.[125,128]

Even small rises in blood creatinine level are associated with an increase in mortality,[129-131] and severity of AKI is correlated with increasing mortality.[132,133] In a mixed population of 2901 critically ill patients in the FINNAKI study, 90-day mortality ranged from 29% to 39% with advancing AKI stages.[133] Poukkanen et al.[43] showed that in severe sepsis AKI developed in 53%, and the 90-day mortality of the patients with AKI was 38% compared with 25% in those without.

Development of AKI is often multifactorial. Several large studies have revealed that age, hypotension for any reason, sepsis or septic shock, cardiogenic shock, mechanical ventilation, coagulation disorders, need for and use of vasoactive drugs,

certain nephrotoxic agents and coexistence of other organ failures predispose to AKI.[133-135]

2.5.3 Microvascular thrombosis versus local cell dysfunction

Historically, sepsis-associated DIC and microvascular thrombosis were claimed to result in, rather straightforwardly, obliteration of blood circulation of different organs, and thus, multiple organ dysfunction.[56,65] Several studies on autopsy cases of DIC have revealed widespread microthrombosis in multiple organs.[136-139] However, in the studies of Watanabe et al.[138] and Tanaka et al.,[139] DIC was defined on a pathological basis. In addition, the studies revealed numerous patients with clinically suspected DIC and no thrombosis (67/109 cases),[139] as well as those with microthrombosis without clinical suspicion of DIC (38/51 cases).[138]

Recent reviews assemble data on experimental and human studies, which demonstrate the connection between altered coagulation and development of organ dysfunction.[140,141] In baboon and rabbit models of lipopolysaccharide (LPS)-induced DIC, administration of TF-inhibitor and inhibition of PAI-1 could prevent acute lung injury and renal fibrin deposition.[142-145] In a murine model, inactivation of t-PA and u-PA genes led to an increased risk of fibrin deposition in many organs and higher incidence of venous thrombi.[146] In a rodent model of AKI, blocking of PAR-2, a molecule with well-known interactions between inflammation and coagulation, inhibited formation of renal fibrin deposits, but no attenuation occurred in renal dysfunction.[147] Also, complete blocking of factor Xa failed to prevent baboons from developing organ damage and dying from experimental *Escherichia coli* sepsis, suggesting that other inflammatory aspects may be responsible for negative outcome.[148] In humans, evidence originates from studies on patients with severe sepsis; DIC contributes to the development of organ dysfunction and is associated with increased mortality.[81,149,150]

Despite widespread activation of coagulation, microthrombosis is likely not a predominant factor in the alterations of microcirculation in sepsis.[151] Recently, Gomez et al.[152] presented their unified theory of sepsis-induced AKI. In brief, inflammatory danger signals launch the adaption of tubular cells; microvascular dysfunction and inflammation amplify this process, and mitochondria initiate cell survival process at the expense of renal function.

Pathophysiology of AKI is still not fully understood, and even histological findings remain controversial. Although many conditions associated with AKI may manifest as global or local ischaemia, hypotension or hypoperfusion, septic AKI more probably arises from cell-based factors. Mariano et al.[153] have shown that plasma, derived from septic patients with AKI, induced apoptosis, reorganized the cytoskeleton and altered the cell polarity in tubular cell culture.

A recent systematic review evaluated both human and experimental data and found that acute tubular necrosis was rather uncommon.[154] Septic AKI, induced by intravenous infusion of *Escherichia coli*, did not present any macroscopic histopathological findings in sheep as compared with controls.[155] In mice, caecal

ligation and puncture induced only minimal acute tubular necrosis, but tubular cell apoptosis was very common and correlated with renal dysfunction.[156]

Lerolle et al.[157] studied post-mortem kidney biopsies of 19 consecutive patients who died of septic shock and AKI. Fibrin depositions were present in the renal capillaries in 42%, complete or partial thrombi in the afferent artery in 21% and glomerular capillary thrombi in only one patient. Other findings were signs of tubular cell apoptosis in all patients, tubular lesions and only minimal inflammation in the interstitium. Remarkably, patients with and without overt DIC had fibrin depositions equally.[157] By contrast, Takasu et al.[158] have questioned a predominant role of apoptosis in the formation of tubular injury. Instead, they revealed transient injury and focal necrosis of tubular cells, mitochondrial injury and signs of tubular regeneration.

2.5.4 Natural anticoagulants in preventing organ dysfunction

The idea of preventing organ dysfunction and thereby improving outcome by restoration of disturbed coagulation by limiting thrombin generation with natural anticoagulants has unfortunately led to disappointments in numerous clinical trials. Supplementation of recombinant TFPI (OPTIMIST study), AT (Kybersept study) and aPC (PROWESS and PROWESS SHOCK studies) in severe sepsis could not reduce 28-day mortality in large, clinical, multi-centre studies.[159-162] These trials have been criticized for overly heterogeneous patient populations, rigid dosing, suboptimal time frames and coincidental administration of other anticoagulants.[163,164] However, quite recently, phase IIb trial on soluble thrombomodulin (s-TM) in severe sepsis patients with concomitant DIC provided promising results, and a phase III trial is ongoing.[165] Other studies on s-TM in haematologic patients with DIC and patients with severe sepsis have revealed that s-TM may decrease mortality and organ dysfunction by resolving DIC.[166-168] TM has both anticoagulant and cytoprotective function. In addition to activation of PC, which possesses well-known anti-inflammatory and anti-apoptotic functions, TM binds harmful HMGB1 and histones and contributes to the inactivation of complement.[24,169-172]

2.5.5 Histones

Histones are proteins normally located in the cell nucleus. Two histone H2A-H2B dimers and one histone H3-H4 tetramer form a condensed, disc-shape structure, a nucleosome, which folds nuclear DNA around this complex by a linker histone H1. The main functions of nucleosomes are to organize and stabilize DNA strands, to regulate transcription and replication and to participate in the DNA repair process.[173,174]

Histones may access extracellular space and circulation by rupture of cell and nuclear membranes in cell death or by active secretion. In the healthy body, a small amount of apoptosis, an organized cell death, occurs continually throughout life.[175] Apoptosis is responsible for cell renewal and tissue homeostasis. In apoptosis, the

cell membrane retains its integration, and apoptotic cells package cell-derived constituents and release them to the extracellular space. In necrosis (also known as oncosis), cell death is unorganized; the cell membrane disrupts and the cell releases intracellular contents into extracellular space, and various enzymatic reactions begin to digest the compounds. Nowadays, also mixed forms of cell deaths are recognized.[173]

In inflammation, proinflammatory cytokines may promote necrosis of activated neutrophils (NETosis) and other leukocytes (ETosis).[176,177] This process spreads intranuclear compounds, including nucleosomes and histones, to the extracellular space, where they form neutrophil extracellular traps (NETs). These components of innate immunity are responsible for killing micro-organisms.[178]

Extracellular histones may appear as small complexes with DNA, mono- and oligonucleosomal fragments, larger DNA-bound fragments, complexes with NETs and free.[173,179] Histones are positively charged ions and bind easily to negatively charged ions, also others than DNA: C-reactive protein (CRP),[180] lipopolysaccharide (LPS),[181] high-mobility group box 1 (HMGB1)[182] and heparin sulphate proteoglycans.[183,184]

Extracellular histones have several deleterious effects. Most importantly, extracellular histones damage host cells, and thus, may cause organ dysfunction in inflammatory diseases. Mice challenged with histones developed fatal changes in their lung tissue.[185] Recently, Allam et al.[186] showed that injection of histones into the renal artery resulted in widespread necrosis of the renal cortex and medulla, whereas inhibition of histones with neutralizing antibody decreased AKI in LPS-induced endotoxemia in mice. At the moment, it is widely accepted that histones may play a major role in development of septic AKI.[187]

Several studies have linked extracellular histones to disturbed coagulation. Histone H4 is a potent promoter of platelet aggregation, and it has caused thrombocytopenia in murine sepsis models.[170,188,189] Studies in the critically ill have presented more conflicting results. Nucleosomal histone-complexed DNA (hcDNA) levels have been associated with a degree of coagulopathy in patients with severe trauma.[190,191] In severe sepsis, one study has reported an association between hcDNA and markers of coagulopathy,[192] and another between extracellular histones H4 and decline in platelet count.[193] Quite recently, a mixed population with overt DIC (the majority of whom were not critically ill patients) was shown to have higher levels of circulating hcDNA and double-stranded DNA than patients without DIC. HcDNA also correlated with D-dimer and predicted 28-day mortality with an area under curve (AUC) of 0.700. [194] **Table 5** summarizes experimental sepsis studies with special reference to coagulation. **Table 6** presents studies on histones in the critically ill.

2.5.6 High-mobility group box 1

A non-histone chromatin-associated protein, HMGB1, acts as a strong proinflammatory cytokine and a late mediator of lethal consequences in severe sepsis when released into the intravascular space.[195,196] In inflammation, several

immune cell types actively secrete HMGB1.[197] However, a controversy long existed about whether necrotic cells only passively release HMGB1 or whether HMGB1 release also occurs in apoptotic cell death.[198,199] Extracellular HMGB1 binds to four receptors: receptor for advanced glycation end-products (RAGE), toll-like receptors 2 and 4 (TLR2 and TLR4) and syndecan-1.[197] Of these, TLR4 is a primary receptor that mediates proinflammatory functions.[200] Once released, HMGB1 concentration stays elevated for days, which makes it an intriguing target for inhibitor trials.[196,201] HMGB1 enhances inflammatory reaction by activating nuclear factor-κB and up-regulating TNF-α.[202] It also interacts with coagulation and may contribute to organ failure. Ito et al. [203] showed that in a murine DIC model, mice developed more excessive glomerular fibrin deposition if thrombin was administered with HMGB1. In critically ill patients suspected of having DIC, HMGB1 levels correlated with DIC and SOFA scores. [204]

Table 5. Experimental studies on nucleosomes and histones in severe inflammation models.

Study	Design	Results
Xu 2009 [185]	Murine and baboon sepsis models with histones, LPS, histone antibodies, aPC	• Extracellular histones cytotoxic to endothelium • aPC degrades histones • aPC may degrade also DNA-bound histones • Toxicity mainly due to H3 and H4 • In a baboon model, H3 was associated with onset of AKI • aPC injection of recombinant aPC rescued all histone-challenged mice • Histology: microvascular thrombosis, fibrin deposition, collagen accumulation
Fuchs 2011 [188]	Murine model of sepsis; murine and human blood, platelets and plasma; histological analysis	• Histones induced platelet aggregation in the presence of fibrinogen (more) or plasma (less) • H4 more potent than H1, H2A, H2B and H3 • Histones induced thrombocytopenia • Histones were lethal also in platelet-depleted mice • Heparin inhibited histone-platelet interaction
Lam 2013 [189]	Blood from healthy volunteers Platelet aggregometry Challenge to H4 and albumin	• Histone H4 induced aggregation of washed platelets • Plasma inhibited aggregation • Albumin-depleted plasma inhibited aggregation less than normal plasma
Nakahara 2013 [170]	Murine model with histone challenge	• Histones caused thrombocytopenia • Citrate inhibited platelet aggregation • Platelet depletion protected from death, but the effect diminished over time, suggesting other mechanisms instead of mere thrombosis • Recombinant thrombomodulin (rTM) protected mice against fatal pulmonary embolism
Abrams 2013 [205]	Murine model with histone and CRP challenge Sera from patients with trauma, pancreatitis and severe sepsis	• CRP inhibited histone-induced endothelial damage and platelet aggregation • Patient sera with either endogenous or exogenous CRP elevation inhibited histone-induced endothelial toxicity
Kowalska 2014 [206]	Murines challenged with histones, platelet factor 4 (PF4) and heparinoids	• Histone challenge increased aPC formation • In sepsis and severe sepsis added heparinoids behave differently depending on histone and PF4 levels: in severe sepsis, non-anticoagulant heparinoid increases endogenic aPC production
Iba 2014 [207]	Rodent LPS-model with antithrombin (AT) and/or rTM substitution	• Combination therapy (AT+rTM) reduced histone H3 and cell-free DNA levels; less organ damage • Fewer dead cells in combination therapy group

LPS, lipopolysaccharide; aPC, activated protein C; AKI, acute kidney injury; CRP, C-reactive protein; AT, antithrombin; rTM, recombinant thrombomodulin.

Table 6. Human studies on nucleosomes and histones in the critically ill.

Study	Patients	Design	Results
Zeerleder 2003 [208]	n=15 with fever n=15 with SIRS n=32 with severe sepsis n=8 with septic shock	Nucleosomes, cytokines, complement, coagulation factors at admission	Nucleosome levels ↑ in 81%, correlated with severity of inflammation. No correlation with cytokines and coagulation tests
Chen 2012 [209]	Surgical ICU Cohort 1: n=45 sepsis n=29 controls Cohort 2: n=70 sepsis n=21 controls	Nucleosomes, within 24 hours of admission	Sepsis vs. severe sepsis vs. septic shock; 35% with sepsis Nucleosomes predicting sepsis: ROC AUC 0.67 (0.55-0.79), independent predictor of sepsis No correlation with sepsis severity or interleukins Correlation with APACHE II
Kutcher 2012 [190]	n=132 critically injured trauma patients	Plasma samples at admission and at 6 hours Histone levels as nucleosomes (Cell death detection ELISA^{plus}®)	Patients with higher histone level has higher trauma score, lower GCS, more MOF, and acute lung injury, longer need for ventilator support, higher mortality. Correlation with coagulation assays
Zeerleder 2012 [210]	n=38 children with meningococcal sepsis	Sub-study of PC supplementation study	Nucleosomes elevated in non-survivors PC treatment had no effect
Johansson 2013 [191]	n=80 trauma patients	hcDNA, biomarkers of coagulation, endothelial injury at admission	Higher hcDNA associated with higher trauma score, D-dimer, tPA. APTT ↑,PAI-1 ↓, F 1+2 ↓ Enhanced inflammation and endothelial damage
Ostrowski 2013[192]	n=9 experimental endotoxemia n=20 severe sepsis	Coagulation tests, adrenaline, noradrenaline, syndecan-1, hcDNA	sTM, PC, PAI-1 and hcDNA strongest correlation with disease severity, coagulopathy and shock hcDNA correlated with lactate

Table 6 (continued). Human studies on nucleosomes and histones in the critically ill.

Study	Patients	Design	Results
Ekaney 2014 [193]	Cohort 1: n=15 severe sepsis Cohort 2: n=19 abdominal sepsis Controls: n=48 wo sepsis, with MOF, small trauma, multiple trauma, healthy	Samples: On day 1, 3 and 5 (cohort 2) Tests: Histone H4, aPC, cytokines, histone stimulation assays, degradation	H4 elevated in sepsis vs. ICU controls (also MOF) Multiple trauma 3-fold level Platelets ↓ <-> H4 ↑ Non-survivors had higher H4. SOFA and white blood cells predicted H4 level aPC: inverse correlation with H4 Half-life of histones 4.6 minutes
Kim 2015 [194]	n= 121 malignancies n= 31 sepsis n= 47 other	hc-DNA, double-stranded DNA, coagulation tests. Time-point not given.	53/199 overt DIC hcDNA and double-stranded DNA elevated in overt DIC HcDNA correlated with DIC score and predicted poor prognosis

APACHE, Acute Physiology and Chronic Health Evaluation; aPTT, activated partial thromboplastin time; AUC, area under curve; F1+2, factor 1+2; GCS, Glasgow Coma Scale; hcDNA, histone-complexed DNA; ICU, intensive care unit; MOF, multiple organ failure; PAI-1, plasminogen activator inhibitor-1; PC, protein C; ROC, receiver operating characteristic; SIRS, systemic inflammatory response syndrome; SOFA, Sequential Organ Failure Assessment; sTM, soluble thrombomodulin; t-PA, tissue-type plasminogen activator.

2.6 MATRIX METALLOPROTEINASES (MMPs)

2.6.1 MMP family

MMPs form a growing family of zinc-dependent endopeptidases with structurally uniform features and a capability of degrading extracellular matrix (ECM). Both transcription and secretion of most MMPs are tightly regulated by several intra- and extracellular factors. Cells secrete most MMPs as inactive zymogens, which are activated by many different serine-proteases or other activated MMPs in extracellular space.[211]

Traditionally, MMPs were classified according to their localization or substrates into subgroups: collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11), matrilysins (MMP-7 and -26), membrane-type MMPs (MMP-14, -15, -16, -17, -24 and -25) and others.[212] MMPs may degrade many substrates not only in ECM but also in other locations, thus having much more complicated functions than originally expected.[213] MMPs play a role in several inflammatory conditions,[214,215] chronic kidney fibrosis,[216] wound healing,[217] pulmonary diseases,[218,219] arthritis,[220] dental diseases,[221] tumour formation and progression,[222] atherosclerosis,[223] cardiovascular diseases,[224] and vascular[225] and bone remodelling.[226]

2.6.2 MMP-8

In 1968, Lazarus et al.[227] first discovered MMP-8, a granulocyte-derived enzyme that cleaved collagen. MMP-8 is known as 'neutrophil collagenase' as it is released by activated neutrophils, and for a long time, the only known substrates were collagens (types I, II and III). Activated MMP-8, however, degrades a great variety of molecules: α_2-macroglobulin, substance P, fibrinogen, angiotensins I and II, plasmin C1-inhibitor and many others.[212,228] Several cell types, including neutrophils, macrophages, plasma cells, several epithelial cells, endothelial cells, fibroblasts and myocytes, express MMP-8.[228]

MMP-8 has a bidirectional function in many diseases. MMP-8 can have both pro- and anti-inflammatory functions.[229,230] It can also either promote or inhibit tumour progression in different cancers.[231,232] MMP-8 has been shown to over-express in rupture-prone atherosclerotic lesions, and contribute to the development of lung fibrosis and wound healing.[233-235]

2.6.3 MMPs in sepsis

Several MMPs have been shown to interact with complex inflammatory reactions on many levels.[236] Experimental studies demonstrate that several MMPs are up-regulated in severe infection models, and the presence of MMP inhibitors or

depletion of MMP-coding genes almost uniformly leads to better survival and less organ failure.[230,237-239] **Table 7** summarizes the most relevant studies on MMPs and tissue inhibitors of metalloproteinase (TIMPs) in patients with severe sepsis or in experimental models.

2.6.4 Tissue inhibitor of metalloproteinase-1 (TIMP-1) as a biomarker

Four TIMPs, logically numbered in order of their discovery, inhibit all MMPs by 1:1 molar ratio. TIMP-2, TIMP-3 and TIMP-4 are unspecific inhibitors of all MMPs, whereas TIMP-1 has a lower affinity to membrane type MMP-1, -3 and -5 and MMP-19.[240] In addition to enzymatic MMP-regulating activity, TIMPs have been suggested to contribute to cell growth and differentiation and to also have anti-apoptotic functions.[212] Furthermore, several proteins besides TIMPs inhibit MMPs.[241]

A recent study on sepsis-induced DIC in murine caecal ligation and a puncture (CLP) sepsis model revealed that TIMP-1 increased early after CLP and onset of coagulopathy.[242] Genetic polymorphism of the TIMP-1 gene resulted in a higher TIMP-1 level and increased mortality in patients with severe sepsis[243] TIMP-1 has proved to be a moderate to good predictor of mortality in patients with severe sepsis.[244,245]

Table 7. Experimental and clinical studies on MMPs and TIMPs in sepsis models and in severe sepsis patients.

Study	Objects	Design	Results
Vandenbroucke 2012 [230]	$MMP^{+/+}$ and $^{-/-}$ mice	LPS-endotoxemia, CLP model Renal ischaemia MMP inhibition	Unselective MMP inhibition protected mice from death MMP-8 $^{-/-}$ protected mice (LPS-model). MMP-8 not responsible for vascular disintegrity in organs despite CNS. MMP depletion reduces CNS inflammation. MMP-/- mice: less coagulopathy, kidney damage and MOF.
Rella 2014 [246]	Healthy men (n=44)	LPS-endotoxemia model	MMP-8 AA genotype had a significantly stronger inflammatory reaction than AG and GG genotypes.
Hoffmann 2006 [245]	Severe sepsis (n=37), healthy controls (n=37)	MMP-2, -9, TIMP-1, -2 on day 1	TIMP-1 discriminated non-survivors with an AUC 0.78. MMP-2, -9 and TIMP-2 no association with APACHE II, no difference between survivors and non-survivors.
Lorente 2009 [244]	Severe sepsis (n=192) Controls (n=50)	MMP-9, -10, TIMP-1 (serum) within 2 hours of diagnosis	TIMP-1 and MMP-10 higher in sepsis; TIMP-1 predicted mortality and correlated with coagulation parameters.
Gäddnäs 2010 [247]	Severe sepsis (n=44) Healthy controls (n=15)	MMP-2, -8, -9, TIMP-1 (serum) On days 1, 4, 6, 8, 10	MMP-2 and -8 higher and MMP-9 lower in severe sepsis. No correlations between APACHE, SOFA or mortality and MMP-8.
Tressel 2011 [239]	Severe sepsis (n=50) $Par1^{-/-}$ and $^{+/+}$ mice	Murine model: LPS or CLP	MMP-1 8.7-fold elevation, independent predictor of DIC and mortality. **Mice:** MMP-1 inhibition improved survival PAR-1-dependently. MMP-1-inhibition: less inflammation and DIC.
Lauhio 2011 [248]	Severe sepsis (n=248) Healthy controls (n=10)	MMP-8, -9, TIMP-1 (serum) at ICU admission	MMP-8 and TIMP-1 higher and MMP-9 lower in non-survivors
Yazdan-Ashoori 2011 [214]	Severe sepsis and healthy controls (both n=15)	MMPs-3,-7,-8,-9, TIMPs -1,-2,-4, on days 0-7	All MMPs and TIMPs elevated in severe sepsis; MMP-9 had negative correlation with disease severity.

Table 7 (continued). Experimental and clinical studies on MMPs and TIMPs in sepsis models and in severe sepsis patients.

Study	Objects	Design	Results
Mühl 2011 [249]	Severe sepsis (n=38), healthy controls (n=17)	MMP-2, -9, TIMP-1, -2 on days 1-5.	MMP-2 no change, MMP-9 and TIMP-1 higher. All markers decreased over time.
Solan 2012 [238]	Children with sepsis / septic shock (n=32/98) Healthy controls (n=32) 2. cohort: septic shock (n=180) MMP-8 $^{-/-}$ mice	Human samples at admission and at 48 hours Murine model: CLP	Human: MMP-8 higher in more severe sepsis. Correlation with mortality and number of organ failures MMP-8 $^{-/-}$(knock-out) and MMP-8 inhibited mice had lower mortality
Lorente 2013 [243]	Severe sepsis (n=275)	MMP-9, -10, TIMP-1, and TIMP-1 polymorphism, inflammatory markers on day 1.	TIMP-1 higher in non-survivors and independent predictor of 30-day mortality. Patients with TIMP-1 T-allele had higher TIMP-1 levels and higher mortality.
Martin 2014 [250]	Severe sepsis (n=90) Non-septic controls (n=91)	All: plasma within 24 hours Subgroup: days 1,3 and 7. MMP-1, -2, -3, -8, -9, -10, -13, TIMPs MMP SNPs	MMP-3, -8, -10 and TIMP-2 higher in sepsis. MMP-9 lower. TIMP-1 no change between patient groups. MMP-1 and -13 SNPs associated with sepsis
Lorente 2014 [251]	Severe sepsis (n=295)	TIMP-1, MMP-9 (serum) Days 1, 4, 8.	Non-survivors: TIMP-1 ↑, MMP-9 ↓, TIMP-1/MMP-9 ratio ↑. TIMP-1/MMP-9 ratio correlated with PAI-1, SOFA, platelets, INR, aPTT.
Wang 2014 [252]	N=480 SIRS (n=80), sepsis (n=180), severe sepsis (n=90), septic shock (n=90)	TIMP-1, MMP-9, NGAL at emergency department admission	TIMP-1 predicted 28-day mortality well (AUC 0.845), NGAL well (AUC 0.833), MMP-9 poorly (AUC 0.700). All those independent predictors of 28-day mortality.

APACHE, Acute Physiology and Chronic Health Evaluation; aPTT, activated partial thromboplastin time; AUC, area under curve; CLP, caecal ligation and puncture; CNS, central nervous system; DIC, disseminated intravascular coagulation; ICU, intensive care unit; INR, international normalized ratio; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; MOF, multiple organ failure; NGAL, neutrophil gelatinase-associated lipocalin; PAI, plasminogen activator inhibitor; PAR, protease-activated receptor; SNP, single-nucleotide polymorphism; SOFA, Sequential Organ Failure Assessment; TIMP, tissue inhibitor of metalloproteinase.

2.6.5 MMPs in coagulation

MMPs have many interactions with coagulation. Platelets express and store several MMPs (e.g. MMP-1, -2, -3 and possibly MMP-9) in cytoplasm, granules or on their surfaces.[253-258] In platelets exposed to collagen, surface-bound collagenase, MMP-1, starts to activate PAR-1 and promote platelet aggregation.[259] MMP-1-PAR-1 interaction disrupts the endothelium, thereby increasing vascular permeability and exposure of tissue factor and collagen. In the study of Tressel et al.,[239] MMP-1-depleted mice had a suppressed cytokine storm and less DIC. MMP-2 has been suggested to enhance other pro-aggregatory mechanisms, and prime platelets for aggregation.[260,261] MMP-9, by contrast, may inhibit platelet aggregation by opposing MMP-2 activity.[262]

Although not involved in platelet aggregation, MMP-8 may contribute to the coagulation process at several other stages. Cunningham et al.[263] showed in laboratory conditions that MMP-8 degraded TFPI by a proteolytic mechanism different from collagen cleavage. By degradation, TFPI fragments lost their ability to inhibit FXa, which may have significance also *in vivo*.[264] Belaaouaj et al.[265] demonstrated similar TFPI degradation effect for MMP-7, -9 and -12 in addition to MMP-8. Hiller et al.[266] showed *in vitro* that MMP-8, -12, -13 and -14 could proteolytically degrade fibrinogen, resulting in substantially impaired clot formation. All studied MMPs, except MMP-8, also cleaved and thereby inactivated FXII.[266] In a murine model of endotoxemia, LPS-induced coagulation disturbance was more pronounced in mice with $MMP^{+/+}$ genotype than in MMP-depleted mice.[230]

The fibrinolytic system interacts with MMPs as well.[267,268] MMP-9 may bind to fibrin and become activated by the plasminogen/plasmin system.[267,269] MMP-3, MMP-7 and membrane-type MMP-1 may also degrade cross-linked fibrin.[270]

3. AIMS OF THE STUDY

The main objectives of this study were as follows:

1. To evaluate the incidence of DIC, the structure and validity of the ISTH score and simultaneous plasma antithrombin activity in a) diagnosis of overt DIC and b) prediction of 28-day mortality in a group of unselected critically ill patients (I).

2. To investigate whole-blood coagulation profile with TEM (ROTEM ®) in patients with severe sepsis with or without overt DIC and to assess the applicability of TEM in the diagnosis of overt DIC (II).

3. To investigate the levels of serum MMP-8 and its inhibitor, TIMP-1, and their associations with different coagulation assays in patients with severe sepsis-associated DIC (III).

4. To evaluate whether systemic nucleus-derived histone-complexed DNA and HMGB1 can predict development of thrombocytopenia, development of AKI defined by KDIGO criteria or 90-day mortality in patients with severe sepsis (IV).

4. PATIENTS AND METHODS

4.1 PATIENTS

The Ethics Committee of the Department of Surgery in Helsinki University Hospital approved all study protocols. In Study I, the Ethics Committee waived the need for informed consent. In Studies II-IV, all patients or their next-of-kin gave informed consent. **Table 8** summarizes the patients and the designs of Studies I-IV.

Table 8. Designs of Studies I-IV.

Study	**I**	**II**	**III**	**IV**
Setting	Retrospective cohort study	Prospective pilot study	Prospective pilot study	Prospective cohort study
Study period	1/2002-10/2003	11/2005-2/2007	1/2002-11/2003	09/2011-10/2011
Hospital	Helsinki University Central Hospital	Helsinki University Central Hospital	Helsinki University Central Hospital	17 ICUs in Finland
Patient number (all/ different groups)	N = 494, of whom n = 306 with DIC associated diagnosis	N = 28, of whom n = 16 no DIC n = 12 with DIC	N = 22, of whom n = 13 no DIC n = 9 with DIC	N = 225, of whom n = 75 platelets <100 n = 150 platelets >100
Main inclusion criteria	Adult patients admitted to the ICU	Severe sepsis at admission Age 18-70 years	Adult patients with severe sepsis within 48 hours	Adult patients with severe sepsis within 48 hours
Main exclusion criteria	Readmissions were excluded (n=10)	Anticoagulant therapy Active bleeding at admission	Anticoagulant therapy Haematological malignancy Chronic liver disease	Prior chronic kidney failure RRT started before admission
Healthy controls	-	n = 10	n = 10	-

DIC, disseminated intravascular coagulation; ICU, intensive care unit; RRT, renal replacement therapy.

4.2 STUDY DESIGNS AND MAIN OBJECTIVES

Study I

In this retrospective cohort study, data were collected from all patients admitted to the medical-surgical ICU of Helsinki University Hospital during the 22-month study period. Locally modified ISTH score for overt DIC was calculated daily in those patients who had a diagnosis known to be associated with DIC (sepsis, major trauma, organ destruction, haematological or solid malignancy, severe obstetrical complication, severe hepatic failure or severe toxic reaction). The main aim was to assess constitution and validity of the ISTH score and simultaneous plasma antithrombin activity in the diagnosis of overt DIC and in predicting 28-day mortality in a group of unselected critically ill patients.

Study II

In this prospective pilot study, we collected blood samples from patients with severe sepsis at admission. Thromboelastometric analyses and global coagulation assays were performed on admission to the ICU. We assessed the whole-blood coagulation profile of patients with or without DIC and healthy controls and compared the results of thromboelastometric analyses with global coagulation assays. We evaluated thromboelastometry parameters and their combinations with regard to diagnosis of overt DIC.

Study III

Study III was a prospective pilot study. We collected blood for MMP-8, TIMP-1 and routine coagulation assays from patients with severe sepsis on days 1 (day of admission), 2, 4 and 7. We compared serum MMP-8 and TIMP-1 concentrations in patients with or without overt DIC and healthy controls and assessed correlations with disease severity and traditional coagulation assays.

Study IV

Study IV was a sub-study of a large, multi-centre, prospective, observational cohort study, the FINNAKI Study, on the incidence, risk factors and outcome of AKI in Finnish ICU patients. We collected plasma from 225 consecutive patients who met the criteria for a diagnosis of severe sepsis within 24 hours of admission to the ICU and analysed systemic histone-complexed DNA and HMGB1 levels on admission (n=225) and at 24 and 48 hours (n=49).

4.3 DEFINITIONS AND DISEASE SEVERITY SCORES

In all studies, severe sepsis was defined according to the recommendation by the American College of Chest Physicians/ Society of Critical Care Medicine Consensus Committee: the patients had to have a suspected or confirmed infection with organ dysfunction. Furthermore, at least two or more criteria of SIRS had to be fulfilled.[40]

Acute Physiology and Chronic Health Evaluation II (APACHE) score and Simplified Acute Physiology Score II (SAPS II) were based on the first 24-hour clinical data and chronic health status.[271,272] Sequential Organ Failure Assessment (SOFA) score was calculated daily based on previous 24-hour clinical data.[273] In Study III, we reported SOFA both with and without points from platelet count.

Diagnosis of overt DIC was based on daily calculations of a modified ISTH score for overt DIC (Studies I, II and III).[7,274] The exact cut-off points for blood platelet count and plasma fibrinogen were set as suggested. Quick-type plasma PT was replaced by Owren-type PT (percentage of remaining activity). The PT cut-off values of 60% and 30% were based on clinical practice and were estimated to equal prolonged PT in seconds. As the ISTH criteria lacked an exact suggestion for fibrin-related marker limits, D-dimer cut-off values were selected according to former local distribution of D-dimer in the critically ill. Modified score for overt DIC is presented in **Table 9**.

Table 9. Modified score for overt DIC.

Points:	0	1	2	3
Platelet count / $x10^9$/l	≥100	50-99	<50	-
Owren-type prothrombin time (activity/ %)	≥60	30-60	<30	-
D-dimer concentration/ mg/l	≤2.0	-	2.1-8.0	>8.0
Fibrinogen/ g/l	≥1.0	<1.0	-	-

Acute kidney injury was defined according to the Kidney Disease: Improving Global Outcomes (KDIGO) Foundation guidelines[128]: increase in serum creatinine (SCr) by ≥26.5 µmol/l within 48 hours, or increase in SCr to ≥1.5 times presumed or known baseline within the preceding 7 days, or urine output < 0.5 ml/kg/hour for 6 hours. AKI was further staged to three different groups (1-3) based on the magnitude of the changes in serum creatinine concentration and urine output depending on which criteria yielded a higher stage.

4.4 CLINICAL DATA COLLECTION

For Study I, up to two major intensive care diagnoses were recorded in the computerized database. Patients were further classified into two groups: those with DIC-associated diagnosis and those without. The patients with DIC-associated diagnosis were sub-divided into eight diagnostic groups according to ISTH suggestion for underlying diagnoses.[7] Routine clinical data including daily laboratory measurements, medications, fluids and transfusions were recorded in the computerized ICU database locally in Helsinki University Hospital ICU in Studies I-III. In Study IV, patient demographics, underlying diagnoses, pre- and peri-ICU

physiological and laboratory data and outcome were collected in the database of the Finnish Intensive Care Consortium (Tieto Ltd., Helsinki, Finland). Data on chronic illnesses, medications, organ dysfunctions and implementation of RRT were recorded in the electronic case report forms. Disease severity scores were calculated locally in Studies I-III and based on data collected in the database of the Finnish Intensive Care Consortium in Study IV. Mortality was recorded on day 28 (Studies I-III) or day 90 (Study IV).

4.5 LABORATORY ANALYSES

Blood samples were taken by an arterial cannula after discarding approximately 5 ml of blood. The samples were taken at admission (I-IV) and daily in the mornings thereafter (I, III, IV). Traditional coagulation assays were analysed by the central laboratory of Helsinki University Hospital according to their standards (I-III). Tubes for the coagulation assays had sodium citrate to a final concentration of 3.8% (I, III) or 3.2% (II). Blood samples from healthy controls (II, III) were taken by venepuncture with a light tourniquet from the antecubital fossa. Samples for the thromboelastometric analyses were stored at room temperature until analysis, which occurred within 15 minutes to 4 hours (II). Serum samples for MMP-8 and TIMP-1 analyses were obtained simultaneously with the other laboratory tests (III), centrifuged at 2000 G for 10 minutes, and the supernatants stored at -80°C until analysis. Samples for thrombin-antithrombin-complex (TAT) and prothrombin fragment 1+2 (F1+2) analyses were stored at -80°C (III). Samples for histone and HMGB1 analyses were collected in ethylenediaminetetraacetic acid tubes and centrifuged at 2000 G for 15 minutes. Plasma was stored at -80°C until analysis.

4.5.1 TEM analyses

Thromboelastometric analyses were performed with ROTEM® (Pentapharm) with four parallel measurement channels and a semi-automated pipetting system according to the manufacturer's instructions. Citrated whole blood (300 µl) was pipetted into specifically coated plastic cups and recalcified with 20 µl of 0.2 mol/l $CaCl_2$. In EXTEM test, coagulation was activated by adding 20 µl of TF-containing reagent, and in FIBTEM test, after activation of coagulation with TF, addition of 10 µl of cytochalasin D eliminated platelet function. The tests were allowed to run 120 minutes. We recorded the following ROTEM® parameters: CT, CFT, α-angle, MCF and LI60 at 60 minutes. The difference between EXTEM and FIBTEM MCF demonstrated platelet contribution.

4.5.2 MMP-8 ad TIMP-1 analyses

MMP-8 and TIMP-1 concentrations were analysed in random order and blinded to the clinical state. MMP-8 serum concentrations were measured by time-resolved immunofluorometric assay by using monoclonal MMP-8-specific antibodies 8708

and 8706 (MedixBiochemica, Kauniainen, Finland) for catching and tracing, respectively.[275] The assay buffer contained 20 mmol/l Tris-HCl, pH 7.5, 0.5 mol/l NaCl, 5 mmol/l calcium chloride, 50 µmol/l zinc chloride, 0.5% bovine serum albumin, 0.05% sodium azide and 20 mg/l diethylenetriamine penta-acetic acid. Samples were diluted in assay buffer and incubated for 1 h, washed and re-incubated with tracer antibody labelled using Europium chelate. Fluorescence was measured using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland).

TIMP-1 analysis was carried out by using commercially available two-site ELISA according to the manufacturer's instructions (Biotrak ELISA System; Amersham Biosciences, Buckinghamshire, UK). The assay recognizes total human TIMP-1: free TIMP-1 and MMP-TIMP-1 complexes.

The levels are reported as ng/ml. Detection limits for MMP-8 and TIMP-1 were 0.032 ng/ml and 1.25 ng/ml, respectively. The interassay coefficients of variation for MMP-8 and TIMP-1 are 4.1% and 13.5% (n=12) and intra-assay coefficients of variation 2.5% and 10.1% (n=12), respectively.

4.5.3 Histones and HMGB1

We studied plasma histone-complexed DNA and HMGB1 as uniplicate by commercial sandwich-type ELISA kits, Cell Death Detection ELISA^{PLUS} (Roche Diagnostics GmbH, Mannheim, Germany) and HMGB1 ELISA (IBL International GmbH, Hamburg, Germany). In hcDNA analysis, mouse anti-histone antibody first binds to the histone component of the mono- and oligonucleosomes in the sample. Second, the DNA component of the nucleosomes is labelled with an anti-DNA antibody that can be photometrically determined.[276] HMGB1 ELISA methodology is essentially similar to that of hcDNA-ELISA. The results of hcDNA are reported as relative absorbance units (AU) and HMGB1 results as ng/ml.

4.6 STATISTICAL METHODS

The statistical analyses were performed by using SPSS versions 10.1 (I), 15.0 (II) and 20.0 (III-IV) (SPSS Inc., Chicago, IL, USA) and BMDP 1.1 for Windows (BMDP Statistical Software, Los Angeles, CA, USA) in Study I. ROC analyses in Studies II and IV were performed by MedCalc for Windows versions 9.3.8.0 and 14.12.0, respectively (MedCalc Software, Mariakerke, Belgium).

4.6.1 Sample size considerations

Study I was a retrospective cohort study, which included all patients in the Meilahti ICU during a 22-month period. Studies II, III and IV were preliminary in nature. Thus, we lacked previous data on thromboelastometry findings, MMP-8 and TIMP-1 concentrations, and histone-complexed DNA levels measured by the chosen

method in severe sepsis-associated coagulation disorders. In Study II, we assessed the B-type error by calculating the difference of means with 95% confidence intervals (CIs) between the groups. On the basis of the incidence of DIC in our previous severe sepsis patients being about 30% (unpublished data from Study I), we targeted the patient count to exceed six in the groups of DIC patients (III). In Study IV, we analysed hcDNA and HMGB1 from only 225 of a total of 686 severe sepsis patients for economical and futility reasons. Time series were obtained from 49 patients to observe the kinetics of the substances.

4.6.2 Data presentation

Data were expressed as numbers with percentage or median with interquartile range (IQR) or 95% CI. Normality of the distribution of the data was tested with histograms, skewness and Shapiro-Wilk test.

4.6.3 Comparisons between and within groups

Data were non-normally distributed, and we chose non-parametric statistical methods for all further analyses. Differences in continuous and categorized data were compared with Kruskal-Wallis analysis of variance (multiple groups), Mann-Whitney U test (two groups), Pearson's X^2 test and two-tailed Fisher's exact test as appropriate according to the sample size. Changes in MMP-8 and TIMP-1 concentrations, and hcDNA and HMGB1 during the study period were analysed with Friedman's two-way analysis of variance based on the ranks. The results are reported as Chi square with degrees of freedom and *p* value.

4.6.4 Spearman's correlation

Correlations between MMP-8, TIMP-1, traditional coagulation assays and disease severity (III), and plasma hcDNA and HMGB1 levels (IV) were analysed with non-parametric Spearman's rank correlation coefficient *Rho* (r_s) with corresponding two-tailed *p* value.

4.6.5 Logistic regression analysis

In Study I, step-wise forward multivariable logistic regression analysis assessed the independent predictors of 28-day mortality in the original publication. For this dissertation, logistic regression analysis was re-performed by inputting a reduced set of factors (sex, gender, APACHE II and maximal DIC score) in order to test the statistical design of the other studies.[60,64] In Study IV, we tested factors having independent predictive value on the development of thrombocytopenia, AKI and all-cause 90-day mortality. The factors having $p<0.1$ in univariable regression were

included in the backward multivariable model. Goodness-of-fit was tested by using the Hosmer-Lemeshow test.

4.6.6 Receiver operating characteristics analysis

Receiver operating characteristics (ROC) analysis was used in Studies I, II and IV. ROC curve illustrates performance of a test by plotting true-positive rate (sensitivity, y-axis) against false positive rate (1-specificity, x-axis). The accuracy of the test depends on the area under the ROC curve: Area 1.0 represents an ideal test, whereas 0.5 is worthless. According to Ray et al.,[277] AUC over 0.9 is considered excellent, 0.75-0.9 good and 0.5-0.75 poor. One possibility to calculate an ideal cut-off point for a diagnostic/prognostic test is Youden's index (sensitivity+specificity-1).[278] For that point, we presented sensitivity, specificity, positive likelihood ratio (IV), negative likelihood ratio and positive and negative predictive values (II), as well as the confidence intervals (I, II, IV) and the standard errors for the AUCs (I and II). **Table 10** presents the statistical measures of the performance.

4.6.7 P value

A *p* value of <0.05 was considered significant in all pair-wise comparisons, except in Study III, where a level of <0.01 was selected in Spearman's rank correlation. In all multiple comparisons (II-IV), *p* was corrected by the number of comparisons (Bonferroni correction).

Table 10. Statistical measures of the performance.

	Condition: YES	**Condition: NO**	
Test: positive	**True positive (TP)** correctly identified	**False positive (FP)** incorrectly identified (Type I error)	**Positive prediction value (PPV)** = TP/ all with a test positive
Test: negative	**False negative (FN)** incorrectly rejected (Type II error)	**True negative (TN)** correctly rejected	**Negative prediction value (NPV)** = TN/ all with a test negative
	True positive rate (TPR) **sensitivity** = TP/ all with a condition	**False positive rate (FPR)** = FP/ all without a condition	**Positive likelihood ratio (LR+)** **= TPR/FPR**
	False negative rate (FNR) = FN/ all with a condition	**True negative rate (TNR)** **specificity** = TN/ all without a condition	**Negative likelihood ratio (LR-)** **= FNR/TNR**
			Odds ratio **= LR+/LR-**

5. RESULTS

5.1 CHARACTERISTICS OF PATIENTS (I-IV)

The studies comprised a total of 769 patients. Study I included altogether 494 critically ill patients; 306 patients had a diagnosis known to be associated with DIC. Patient characteristics, disease severity scores, diagnostic groups (Study I) and 28-day mortality are summarized in **Table 11** (whole cohort of Study I) and **Table 12** [sub-cohort of Study I (n=306), and all patients of Studies II and III].

Table 11. Patient characteristics, disease severity scores, 28-day mortality and diagnostic groups of all patients in Study I. The 28-day mortality rate is previously unpublished in these patient groups.

DIC associated critical illness	**Yes** n=306 (62%)	**No** n=188 (38%)
Age	53 (42-64)	57 (50-68)
Sex (M/F)	221 (72%)	131 (70%)
APACHE II	15 (11-20)	12 (9-19)
SOFA day 1	9 (6-12)	6 (4-8)
Mortality day 28	70 (23%)	33 (18%)
Diagnostic group:		
Sepsis or severe infection	179	na
Severe trauma	21	na
Organ destruction	33	na
Malignancy	10	na
Vascular abnormality	46	na
Severe hepatic failure	7	na
Toxic/ immunologic reaction	8	na
Obstetric calamity	2	na
Post cardiopulmonary resuscitation status or cardiac failure	na	148
Miscellaneous	na	40

Values are presented as number of patients (%) or median (interquartile range). DIC, disseminated intravascular coagulation; APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment; na, not applicable.

Table 12. Patient characteristics, disease severity scores and 28-day mortality in patients with critical illness known to be associated with DIC (subgroup of Study I patients) and in all patients with severe sepsis (Studies II and III). Unpublished results regarding age and gender of the patients in Study I.

Study	**I**		**II**		**III**	
Diagnosis	**Any critical illness known to associate with DIC**		**Severe sepsis**		**Severe sepsis**	
	No DIC n=211	**DIC n=95**	**No DIC n=16**	**DIC n=12**	**No DIC n=13**	**DIC n=9**
Age	54 (40-65)	52 (45-63)	44 (39-55)	48 (34-58)	53 (46-64)	56 (47-71)
Sex (M/F)	151 (72%)	70 (74%)	11 (69%)	7 (58%)	7 (54%)	6 (67%)
APACHE II	14 (10-18)	18 (13-23)	16 (13-20)	24 (18-27)	17 (13-20)	20 (15-22)
SOFA Day 1	8 (5-10)	12 (10-14)	9 (7-10)	13 (10-17)	7.5 (6-12)	12 (10-14)
Mortality Day 28	32 (15%)	38 (40%)	0 (0%)	5 (42%)	2 (15%)	4 (44%)

Values are presented as number of patients (%) or median (interquartile range). DIC, disseminated intravascular coagulation; APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment.

In Study IV, the whole FINNAKI study cohort included 918 patients with severe sepsis. Of these, 232 patients were excluded for the following reasons: 123 patients fulfilled the criteria of severe sepsis >24 hours after admission, 69 had chronic renal failure or RRT was started before admission, and 40 patients declined to participate. Of the remaining 686 patients with severe sepsis diagnosis within 24 hours, we studied a cohort of consecutive 225 patients (1.9.2011 - 23.10.2011). Thrombocytopenia was diagnosed in 75/225 patients (33%). Patient characteristics, admission and organ failure data and 90-day mortality are reported in **Table 13**.

In Studies I, II and IV, the patients with coagulopathy, either overt DIC (I, II) or thrombocytopenia (IV), had higher APACHE II [p<0.001 (I) and p=0.014 (II)], higher SAPS II [p=0.001 (IV)], and higher day 1 SOFA scores [p<0.001 (I) and p=0.001 (II)]. In Study III, disease severity scores did not differ between groups. Number of organ failures and proportion of patients in Study IV are presented in **Figure 4**.

Table 13. Patient characteristics, admission and organ dysfunction data and 90-day mortality of the patients in Study IV.

Characteristics	**Platelets ≥ 100 x10^9 /l n = 150**	**Platelets < 100 x10^9 /l n = 75**	***p***
Age	65 (50-74)	67 (57-77)	0.142
Gender (male,%)	94 (63%)	52 (69%)	0.323
Baseline creatinine (µmol /litre)	79.5 (66-93)	78.0 (66-93)	0.855
Septic shock	109 (73%)	67 (89%)	0.004
RRT	11 (7%)	16 (21%)	0.002
Length of hospital stay (days)	12 (7-23)	15 (8-26)	0.388
90-day mortality	36 (24%)	29 (39%)	0.022
Organ failure data			
SAPS II	39 (30-46)	44 (35 – 55)	0.001
Number of organ failures (SOFA stages 3-4)	2 (1-2)	2 (1-3)	0.001
KDIGO AKI (any stage)	63 (42%)	51 (68%)	<0.001
First day of AKI (day)	1 (0-1)	0 (0-1)	0.680
Highest KDIGO stage:			
Stage 1	26 (41%)	20 (39%)	na
Stage 2	18 (29%)	10 (20%)	na
Stage 3	19 (30%)	21 (41%)	na

Values are presented as number of patients (%) or median (interquartile range). RRT, renal replacement therapy; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment; KDIGO, the Kidney Disease: Improving Global Outcomes; AKI, acute kidney injury; na, not available.

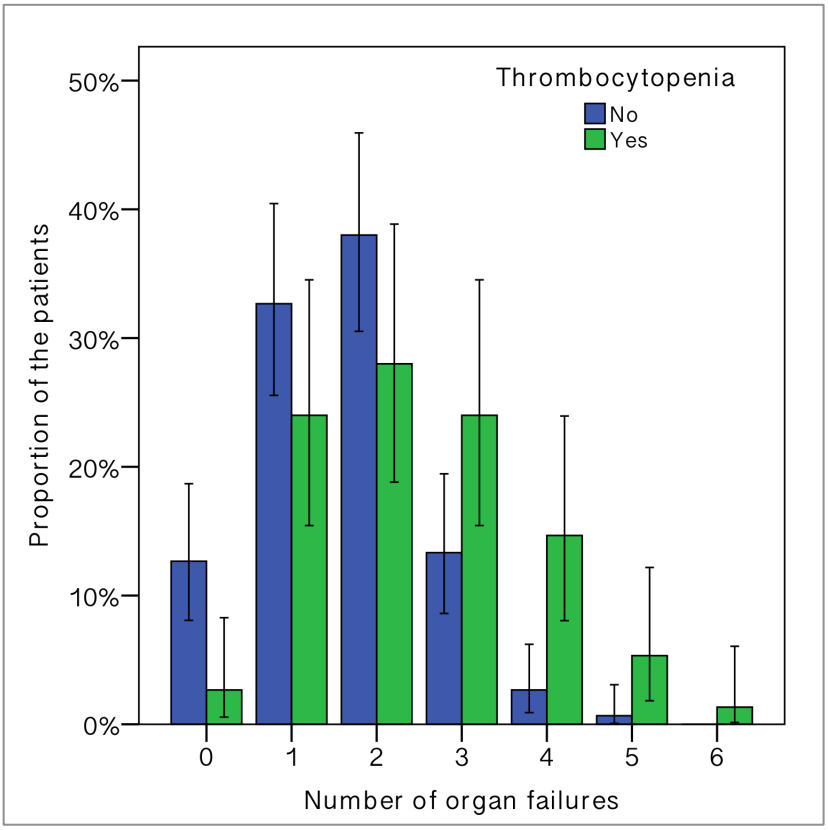


Figure 4. Number of organ failures (SOFA score points 3 or 4) in patients with platelet count above and below 100 x10^9/l (IV).

5.2 ROUTINE BLOOD TESTS IN OVERT DIC (I-III) AND THROMBOCYTOPENIA (IV)

Blood platelet count, plasma PT ratio, D-dimer, fibrinogen, AT, CRP and blood lactate results in patients with and without overt DIC (I-III) and thrombocytopenia (IV) are summarized in **Tables 14** and **15**, respectively. Coagulation test results (except fibrinogen in Study III) differed between groups (all *p*'s <0.01). No difference in plasma CRP between groups existed in Studies I, III and IV. Lactate level was higher in patients with DIC (I) and thrombocytopenia (IV) (p<0.001 for both).

Table 14. Traditional coagulation test results in patients with critical illness known to be associated with DIC (subgroup of Study I patients) and in all patients with severe sepsis (Studies II and III).

Study	**I**		**II**			**III**	
	Any critical illness known to associate with DIC		**Severe sepsis**			**Severe sepsis**	
	No DIC n=211	**DIC n=95**	**No DIC n=16**	**DIC n=12**	**Controls n=10**	**No DIC n=13**	**DIC n=9**
Platelets ($x10^9$/l)	121 (83-179)	34 (21-50)	186 (84-242)	64 (40-96)	210 (193-261)	131 (80-179)	50 (34-81)
PT ratio (%)	50 (38-62)	28 (23-40)	51 (36-74)	34 (28-41)	90 (83-103)	67 (52-77)	34 (24-53)
D-dimer (mg/l)	4.9 (2.4-7.8)	14.9 (7.6-27.7)	1.5 (1.0-2.7)	8.4 (5.5-24.8)	0.1 (0.1-0.2)	1.9 (1.0-4.2)	6.5 (4.8-41.5)
Fibrino-gen (g/l)	6.1 (3.3-7.5)	4.2 (2.5-5.4)	4.9 (3.1-9.5)	2.8 (2.2-5.4)	2.7 (2.4-3.3)	7.0 (5.9-10.6)	5.6 (4.8-7.5)
AT (%)	55 (42-67)	33 (23-42)	44 (36-56)	34 (19-52)	112 (107-118)	56 (41-64)	30 (21-43)
CRP (mg/l)	182 (96-270)	160 (88-223)	na	na	na	310 (233-397)	280 (170-311)
Lactate (mmol/l)	1.4 (0.9-2.1)	3.5 (1.6-6.8)	na	na	na	na	na

Values are presented as number of patients (%) or median (interquartile range). In Study I, coagulation test results refer to the most pathological value of the study period; in studies II and III to admission samples. CRP and lactate were analysed at admission. DIC, disseminated intravascular coagulation; PT, prothrombin time; AT, antithrombin; CRP, C-reactive protein, NA, not available.

Table 15. Laboratory test results in Study IV.

Laboratory test results	Lowest platelets >100 x10^9 /l	Lowest platelets <100 x10^9 /l
Platelet count first 24 hours (x10^9 /l)	230 (166-303)	85 (53-113)
Platelet count lowest (x10^9 /l)	181 (140-250)	53 (24-83
Lactate highest; day of admission or <24 hours prior (mmol/l)	1.9 (1.2 – 3.3)	4.0 (2.5 – 6.9)
C-reactive protein (mg/l)	160 (61-271)	128 (53-242)

5.3 MODIFIED SCORE FOR DIC IN THE DIAGNOSIS OF DIC (I)

In Study I, 306 patients had underlying diagnosis known to be associated with DIC. Of these, 95 patients fulfilled the criteria for DIC during the study period. The incidence of DIC during the study period was 95/494 (19%) in all patients, and 95/306 (31%) in those with appropriate underlying diagnosis.

The 28-day mortality was higher (38/95, 40%) in the patients with overt DIC than in those without DIC (65/399,16%; p<0.001). In the patients with appropriate underlying diagnosis without DIC, 28-day mortality was 32/211 (15%). **Figure 5** demonstrates an increase in 28-day mortality with higher DIC score.

As different components of DIC score were assessed by ROC curves (**Table 16**), platelet count had excellent, PT ratio and D-dimer good and fibrinogen concentration poor ability to discriminate patients with overt DIC. AT discriminated patients well.

Logistic regression analysis revealed only APACHE II score (p=0.003), day 1 SOFA (p=0.003), CRP at admission (p=0.036) and the lowest AT (p=0.004) as independent predictors of 28-day mortality. In a re-performed logistic regression analysis with a reduced set of variables, only APACHE II score and maximal DIC score during the study period independently predicted 28-day mortality (p <0.001 for both) (unpublished data).

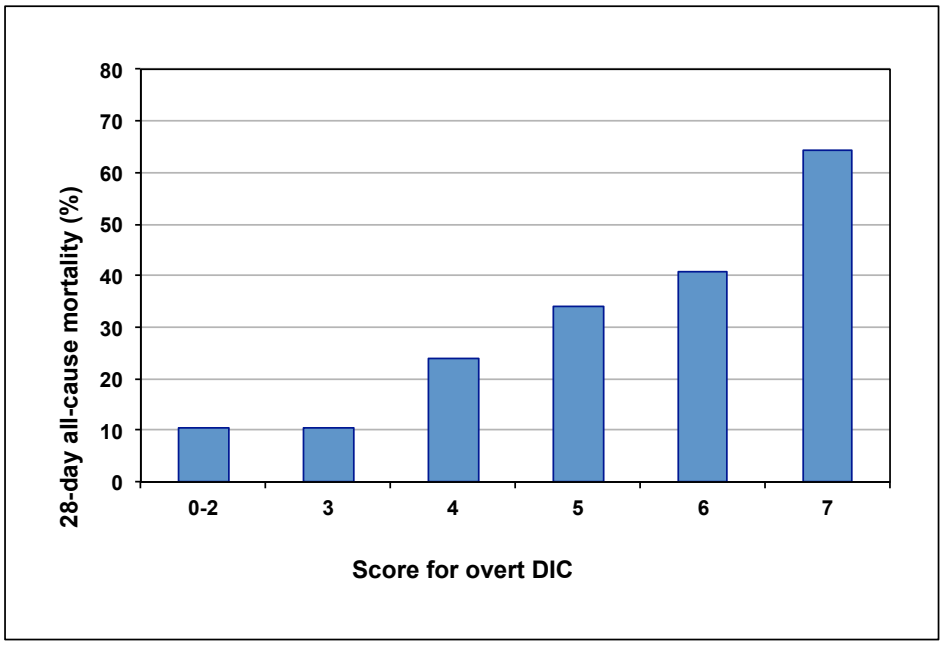


Figure 5. Mortality of critically ill patients and the highest score for overt DIC.

Table 16. ROC curve AUCs with 95% confidence intervals for DIC score components and antithrombin in predicting overt DIC (Study I).

	AUC	95% CI
Platelet count	0.910	0.870-0.950
PT ratio	0.797	0.743-0.851
D-dimer	0.846	0.803-0.890
Fibrinogen	0.690	0.596-0.783
Antithrombin	0.823	0.772-0.974

AUC, area under curve; CI, confidence interval; PT, prothrombin time.

5.4 THROMBOELASTOMETRY IN SEVERE SEPSIS AND DIC (II)

TEM test results are presented in **Table 17**. EXTEM and FIBTEM parameters did not differ in the patients with severe sepsis without DIC as compared with healthy controls. Only EXTEM and FIBTEM MCF had a trend for an elevation, but the change was not significant after correction for multiple comparisons and predefined level of significance (p=0.042 and p=0.034, respectively). Traditional coagulation assays, except for platelet count, showed a simultaneous change towards coagulopathy (**Table 14**).

In overt DIC, EXTEM CFT was prolonged (p=0.002) and α angle (p=0.003) and MCF were decreased (p=0.002) relative to both patients without DIC and healthy controls (all p<0.01). FIBTEM MCF was at the level of healthy controls (**Table 17**). **Figure 6** demonstrates day 1 EXTEM MCF in different study groups as a box plot. **Figure 7** demonstrates appearance of TEM curves in healthy controls, and severe sepsis patients with and without DIC. In severe sepsis with and without DIC, fibrinolysis was inhibited as assessed by LI60 (p=0.01 and p=0.002, respectively). No change occurred between patient groups (**Table 17**).

ROC curves analyses revealed that EXTEM CFT, α angle and MCF discriminated septic patients with and without overt DIC well. None of the parameters was significantly superior in pairwise comparison. A combination of either α angle or CFT with MCF may have increased specificity. **Table 18** demonstrates AUCs, 95% CIs, cut-off values, sensitivities, specificities and positive likelihood ratios for day 1 EXTEM CFT, α angle and MCF.

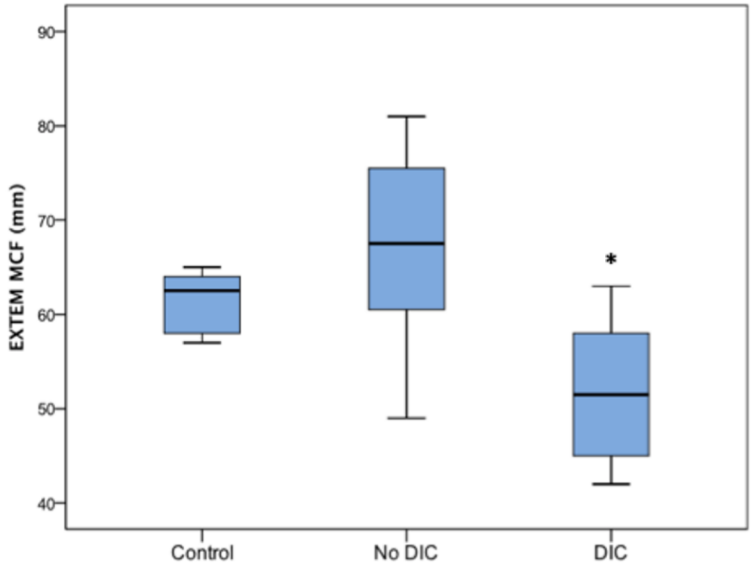


Figure 6. EXTEM MCF (mm) in severe sepsis patients with and without overt DIC and in healthy controls.

Table 17. Thromboelastometry test results on day 1.

	Healthy controls n=10	**Severe sepsis without overt DIC n=16**	**Severe sepsis with overt DIC n=12**	***Overall p***
EXTEM				
CT	64 (54-73)	72 (49-125)	78 (60-104)	0.318
CFT	88 (78-102)	73 (67-143)	184 (101-285) §¶	0.004 *
MCF	63 (58-64)	68 (60-76)	52 (45-58) §¶	<0.001 *
α-angle	72 (70-74)	76 (66-78)	58 (46-70) §¶	0.003 *
LI60	90 (87-92)	96 (91-98) †	97 (93-98) ¶	0.006 *
FIBTEM				
CT	63 (60-70)	68 (52-94)	71 (60-87)	0.470
MCF	15 (13-17)	23 (14-36)	14 (11-21)	0.033 *
LI60	94 (93-96)	98 (96-98)	97 (95-98)	0.093

Data are reported as median (interquartile range). * $P < 0.05$: all groups, † $P < 0.01$: severe sepsis patients without overt DIC vs. healthy controls; ¶ $P < 0.01$: severe sepsis patients with overt DIC vs. controls; § $P < 0.01$: severe sepsis patients with vs. without overt DIC. CT, clotting time; CFT, clot formation time; MCF, maximal clot firmness; LI60, lysis index at 60 minutes; DIC, disseminated intravascular coagulation.

Table 18. Receiver operating characteristic curve AUCs with 95% confidence intervals, cut-off values and positive likelihood ratios for EXTEM parameters in predicting overt DIC.

	CFT	**α**	**MCF**	**α + MCF**
AUC	0.815	0.828	0.891	
95% CI	0.624-0.935	0.639-0.943	0.715-0.975	
Cut-off value	>74 seconds	≤72º	≤63 mm	≤72º and ≤63 mm
Sensitivity	100%	100%	100%	100%
Specificity	56%	56%	69%	75%
LR+	2.3	2.3	3.2	4.0

AUC, area under curve; CI, confidence interval; LR+, positive likelihood ratio; CFT, clot formation time; MCF, maximal clot firmness.

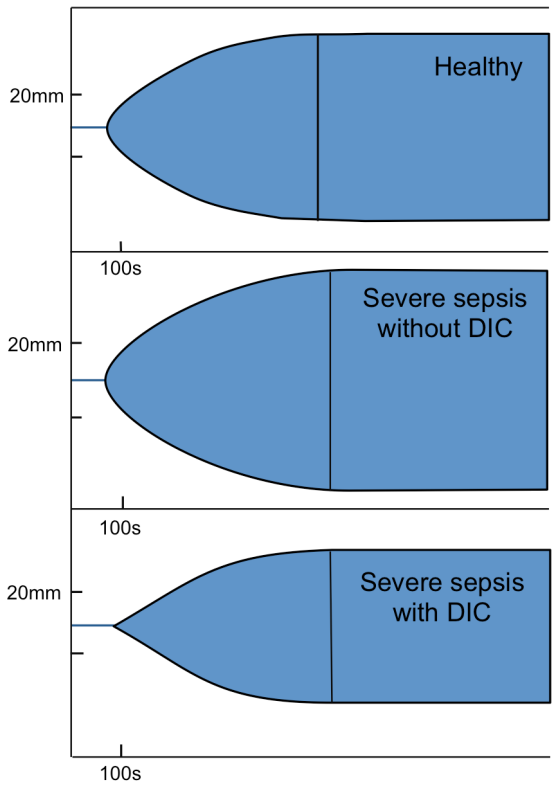


Figure 7. Characteristic TEM curves for healthy controls, and severe sepsis patients without and with DIC based on median values of MCF, CT and CFT in different study groups.

5.5 MMP-8 IN SEVERE SEPSIS AND OVERT DIC (III)

Figure 8 demonstrates MMP-8 serum concentration in study groups during the whole study period. MMP-8 was elevated in patients with severe sepsis as compared with healthy controls (p<0.001 at each time-point). Patients with overt DIC differed from those without DIC only on day 2 [702 (IQR 540-1042) ng/ml vs. 246 (IQR 58-550) ng/ml, p=0.020]. MMP-8 significantly decreased during the study period in both patients with DIC (p<0.001) and those without (p<0.01).

MMP-8 had a strong correlation with TIMP-1. MMP-8 concentration correlated negatively only with platelet count on day 2 [r= -0.626, p=0.003). On day 1, correlation with platelet count (r= -0.483, p=0.026) did not reach the predefined level of significance. MMP-8 had no correlation with disease severity scores, CRP or white blood cell count.

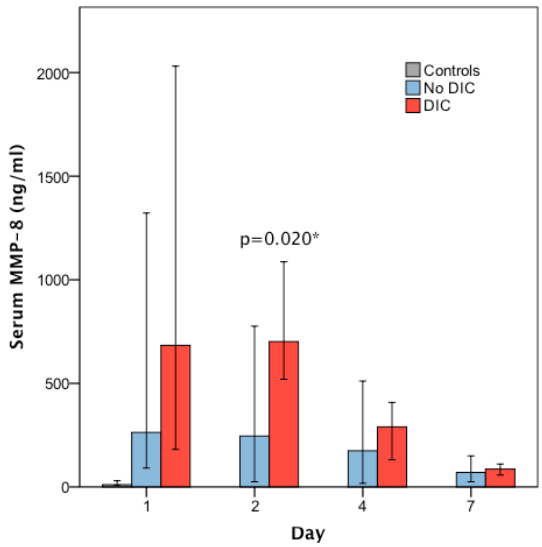
5.6 TIMP-1 IN SEVERE SEPSIS AND OVERT DIC (III)

Figure 8 demonstrates TIMP-1 concentrations in different study groups with significant p values. TIMP-1 concentration was higher in septic patients than in controls (p<0.001). Patients with overt DIC had higher TIMP-1 level on days 1 and 2, but not thereafter [day 1: 1034 (IQR 793-1102) ng/ml vs. 542 (449-836) ng/ml, p=0.014; and day 2: 788 (601-1074) ng/ml vs. 447 (357-545) ng/ml, p=0.006] as compared with those without DIC. In all patients, TIMP-1 levels declined during the study period (p<0.001 for those with DIC, p=0.002 for those without DIC).

TIMP-1 had a negative correlation with platelet count on day 2 (r= -0.641, p=0.002), with PT ratio on days 1 and 2 (r= -0.760, p<0.01; r= -0.620, p=0.005) and with PC on day 2 (r= -0.586, p=0.008). TIMP-1 correlated positively with D-dimer on day 2 (r= 0.663, p=0.002). TIMP-1 had a positive correlation with SOFA score on days 1 and 2 (r= 0.634, p=0.003; r= 0.634, p=0.010).

Figure 9 presents unpublished results on ROC curve for admission TIMP-1 in predicting overt DIC. TIMP-1 discriminated patients with overt DIC from those without DIC with an AUC of 0.894 (95% CI 0.775-1.000, p=0.001). Due to small sample size, no cut-off limit was calculated.

8 a)



8 b)

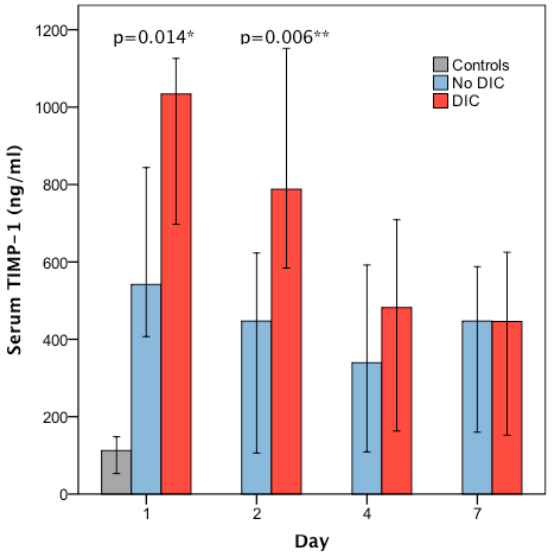


Figure 8. MMP-8 (a) and TIMP-1 (b) concentrations in severe sepsis with and without overt DIC, and healthy controls. Data presented as medians with 95% confidence intervals. **P*<0.05 and ***p*<0.01 in comparing patients with and without DIC.

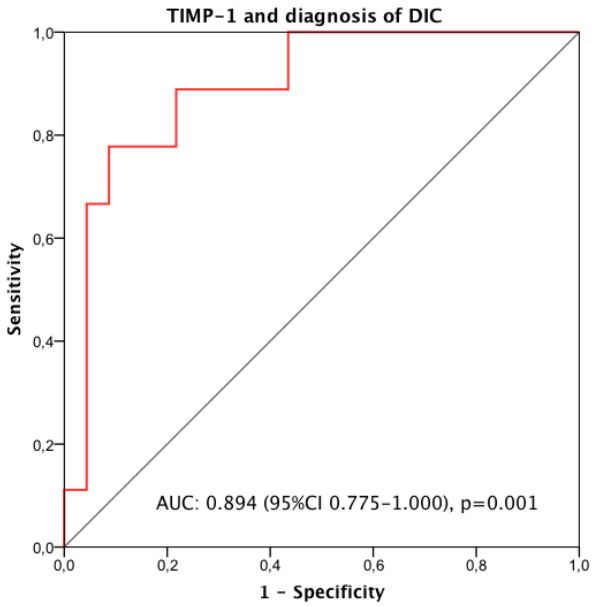


Figure 9. ROC curve for TIMP-1 in prediction of DIC. Unpublished results.

5.7 HISTONES AND HMGB1 IN SEVERE SEPSIS AND ORGAN FAILURE (IV)

Table 19 summarizes plasma levels of hcDNA and HMGB1 in patients with and without thrombocytopenia (defined as platelet count <100 $x10^9$/litre) or AKI of any KDIGO stage, and in those who died within 90 days. As the patients were divided into four groups according to the quartiles of hcDNA level, the relative number of patients with AKI increased along with elevating hcDNA level (incidence of AKI was 43%, 39%, 55% and 66% for quartiles I to IV, respectively, p=0.015).

Figure 10 demonstrates kinetics of hcDNA and HMGB1 during the study period of 48 hours. The incidence of AKI (49%), thrombocytopenia (43%), and 90-day mortality (18%) did not differ from the whole study cohort (p 0.789, 0.110 and 0.066, respectively). HcDNA levels increased in 26/49 (53%), decreased in 22/49 (45%) and remained unchanged in 1 patient. HMGB1 concentration increased in 17/49 (35%), decreased in 27/49 (55%) and remained unchanged in 5/49 patients (10%). Direction of a change in hcDNA or HMGB1 did not predict thrombocytopenia or AKI of any stage in logistic regression analysis (p>0.05 for all).

Maximal hcDNA correlated moderately with HMGB1 (r_s 0.496, p<0.001), and weakly with highest lactate (r_s 0.298, p<0.001), maximal SOFA score (r_s 0.399, p<0.001) and SAPS II (r_s 0.203, p=0.002). A weak negative correlation occurred between hcDNA and lowest platelet count (r_s -0.266, p<0.001).

Table 20 shows the results from multivariable logistic regression analysis. Higher hcDNA, age, lower BMI and lactate were independent predictors of thrombocytopenia, whereas HMGB1 had no independent predictive value. HcDNA did not independently predict AKI of any severity. HMGB1, however, predicted both development of AKI stage 3 and 90-day mortality [1.141 (1.030-1.263), p=0.012]. **Figure 11** and **Table 21** present ROC curves and respective AUCs with 95% CIs, sensitivities, specificities and positive likelihood ratios for hcDNA and HMGB1 in predicting thrombocytopenia, AKI of any stage and, specifically, AKI stage 3.

Table 19. Plasma HcDNA and HMGB1 levels in patients with thrombocytopenia, AKI of any stage and adverse outcome.

	hcDNA (AU)		**HMGB1 (ng/ml)**	
		p		*p*
Lowest platelet count <100x10^9 /l				
Yes (n=75)	0.258 (0.100-0.573)	0.002	1.559 (0.550-4.530)	0.009
No (n=150)	0.154 (0.073-0.285)		0.984 (0.446-1.837)	
AKI of any stage				
Yes (n=114)	0.306 (0.146-0.741)	<0.001	1.216 (0.676-3.776)	0.009
No (n=111)	0.150 (0.0638-0.309)		0.921 (0.371-1.719)	
Dead on day 90				
Yes (n=65)	0.250 (0.139-0.718)	0.003	1.389 (0.671-4.734)	0.009
No (n=160)	0.172 (0.066-0.389)		1.041 (0.421-2.005)	

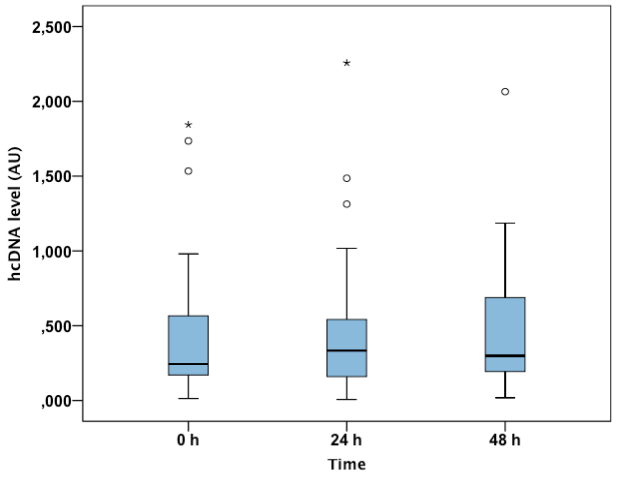
Data are reported as medians with interquartile ranges and *p* values. AKI, acute kidney injury; hcDNA, histone-complexed DNA; HMGB1, high-mobility group box 1.

Table 20. Independent predictors of thrombocytopenia, AKI of any KDIGO stage and AKI KDIGO stage 3.

	Platelets <100x10^9 /litre	**AKI, any KDIGO stage**	**AKI, KDIGO stage 3**
hcDNA	5.433 (1.989-14.837) **	ns	ns
HMGB1	ns	ns	1.099 (1.014-1.191) *
Age	1.032 (1.009-1.056) **	ns	ns
BMI	0.848 (0.775-0.927) **	ns	ns
Diabetes	ns	2.426 (1.188-4.460) *	3.568 (1.542-8.259) **
SAPS II	ns	1.032 (1.003-1.063) *	ns
Lactate	1.189 (1.067-1.324) **	1.183 (1.040-1.346) *	1.089 (1.007-1.178) *

Values are reported as odds ratios (ORs) with 95% confidence interval (95% CI). SAPS II score is assessed without renal components and age. * p <0.05 and ** p<0.01. BMI, body mass index; SAPS, Simplified Acute Physiology Score; hcDNA, histone-complexed DNA; HMGB1, high-mobility group box 1.

10 a) HcDNA levels (AU) at different time-points.



10 b) Plasma HMGB1 concentrations (ng/ml) at different time-points.

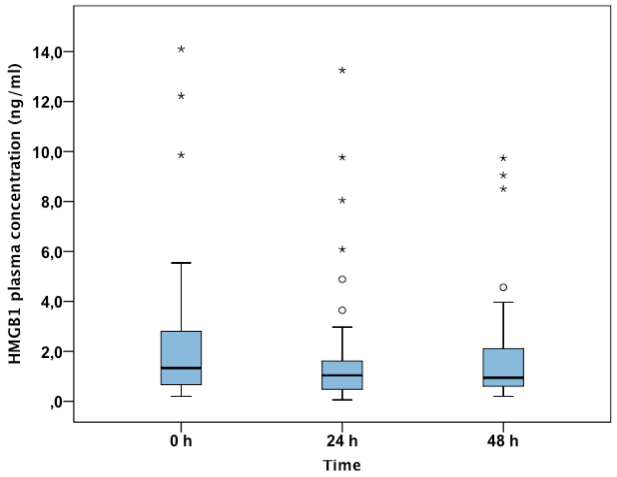
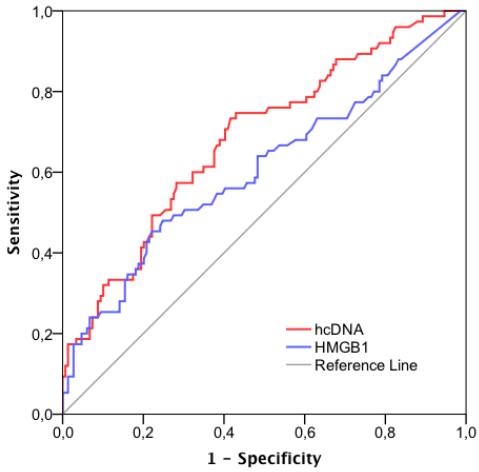
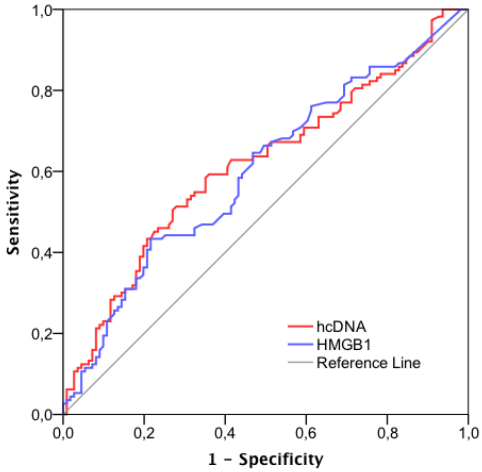


Figure 10. Kinetics of plasma hcDNA and HMGB1 in severe sepsis (a subcohort of 49 patients including patients with both thrombocytopenia and AKI). No change occurred in hcDNA level during the first 48 hours (p=0.660) (**Figure 10a**). HMGB1 concentration was higher on admission (p=0.048) (**Figure 10b**).

11 a) Thrombocytopenia



11 b) AKI of any stage



11 c) AKI stage 3

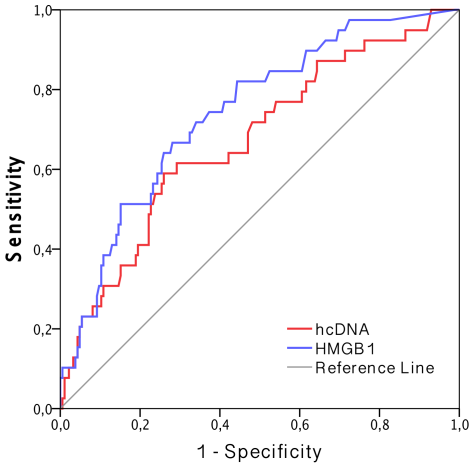


Figure 11. ROC curves for hcDNA and HMGB1 regarding thrombocytopenia (a), AKI of any KDIGO stage (b) and AKI KDIGO stage 3 (c).

Table 21. Receiver operating characteristic curve data for predicting AKI of any KDIGO stage and thrombocytopenia.

	AUC	95% CI	Cut-off point	Sensitivity (%)	Specificity (%)	LR+
AKI, any stage						
hcDNA	0.620	0.553-0.684	>0.244	50.9	73.0	1.88
HMGB1	0.601	0.533-0.665	>1.782	43.3	78.4	2.01
AKI, stage 3						
hcDNA	0.673	0.607-0.734	>0.333	60.0	74.1	2.31
HMGB1	0.742	0.679-0.798	>1.628	66.7	71.9	2.37
Platelets <100 x10^9 /l						
hcDNA	0.679	0.614-0.740	>0.172	74.7	56.7	1.72
HMGB1	0.607	0.540-0.672	>2.167	45.3	77.9	2.05

Cut-off point for hcDNA reported as AU and for HMGB1 as ng/ml. AKI, acute kidney injury; hcDNA, histone-complexed DNA; HMGB1, high-mobility group box 1; AUC, area under curve; CI, confidence interval; LR+, positive likelihood ratio.

6. DISCUSSION

6.1 INCIDENCE OF THROMBOCYTOPENIA AND DIC

Study I conducted in a multidisciplinary ICU of a tertiary care hospital showed that incidence of overt DIC in unselected critically ill patients was 19% and in those with appropriate underlying diagnosis 31%. The smaller studies (II and III) included only patients with severe sepsis. Expectedly, incidence of DIC was higher, exceeding 40%. In Study IV, 33% of the patients developed thrombocytopenia (defined as platelet count below 100 $x10^9$/litre) during the first 5 days in the ICU.

Different study populations plausibly explain the differences in DIC incidences. In Study I, we did not separately analyse incidence of DIC in patients with severe sepsis; the proportion might have been higher in that sub-group. Study IV, in contrast to the others, was a multi-centre study, including patients from all participating ICUs during the recruitment period. Study IV best reflects the incidence of severe sepsis-related coagulation disorders in a mixed population.

Large multi-centre studies (Finnsepsis study and subpopulation of FINNAKI study including all patients with severe sepsis) in Finland have reported concordant incidences of severe thrombocytopenia, defined as platelet count <50 $x10^9$/litre, to range from 14.5% to 22%.[43,47] Calculation of DIC score, however, requires measurements of platelet count, PT and at least D-dimer, and these tests do not belong to the daily routines in all patients. In studies from other countries, the incidences of thrombocytopenia vary vastly;[31] in two large trials investigating the potential role of natural anticoagulants in the treatment of severe sepsis, incidences of DIC were (in these selected populations) between 30% and 40%, as defined by ISTH score.[58,59]

6.2 DIC SCORE IN THE DIAGNOSIS OF OVERT DIC

Originally, the development of the DIC score arose from a clinical need for a unified definition. Easily available traditional coagulation tests were included in the score based on previous understanding of their behaviour. Study I demonstrated that of the components in the score platelet count, PT and D-dimer predicted DIC well, whereas fibrinogen seemed to be useless. None of the patients in the study cohort had fibrinogen concentration below 1.0 g/litre. Fibrinogen is an acute phase protein, which can be strongly up-regulated in inflammatory diseases.[279] Since the original suggestion, the meaning of fibrinogen in sepsis-associated DIC has been questioned,[59,78,85] although a study comparing ISTH and JMWH scores and their composition suggested that fibrinogen was not a sensitive, but, instead, a specific DIC marker in patients without an underlying inflammatory diagnosis.[280] Baboon endotoxemia model showed that severity of inflammatory reaction and outcome depended on individual ability to compensate a transient drop of fibrinogen. Those with good compensatory mechanisms survived.[281] In non-ICU DIC originating mainly

from haematological malignancies, high fibrinogen was associated with worse outcome.[78]

Platelet count possessed the best discriminative power compared with the other components of the score. This is hardly surprising given that it is well known that among the coagulation tests platelet count is a strong predictor of organ dysfunction and even outcome.[32,36-38] Platelets decrease until day 4-6, after which they start to increase.[33]

D-dimer or some other fibrin-related marker has a central role in the score. Originally, ISTH did not give suggestions for exact cut-off points, and they were defined as "moderate increase" and "strong increase". D-dimer cut-off limits in Study I are higher than those suggested by two other studies, as they were selected according to former local distribution of D-dimer values (unpublished data).[59,64] Also other cut-offs have been suggested.[282,283] A large number of assays for D-dimer are on the market, and their results vary vastly. The authorities have demanded validation of different D-dimer assays, but because of the multitude of the assays this seems to be an impossible task. The assay used by our central laboratory produces somewhat higher results than the assays on average.[284] In practice, ICU patients almost uniformly have elevated D-dimer regardless of the underlying disease. In Study I, 303 of 306 patients had D-dimer >0.5 mg/l, and if this had been used as a lower cut-off limit in this study instead of >2.0 mg/l, altogether 124 (41%) instead of 95 (31%) of the 306 patients would have fulfilled the diagnosis of overt DIC (unpublished data). Thus, in critically ill patients, diagnosis of DIC is dependent mainly on prolonged PT/ reduced PT ratio and a severity of thrombocytopenia, as virtually all patients present with elevated D-dimer.

The original suggestion for a DIC score by ISTH included PT ranges in seconds. In Nordic countries, PT is measured by Owren-type method for historic reasons, and the results are reported as a PT ratio (percentage).[70] In Study I, we modified PT ranges by replacing them by percentages of approximately the same levels (60% and 30%). Most probably, the majority of studies reporting PT values use the Quick method. The use of Owren-type PT and the limits chosen are, however, considered justified, as assessment of performance of modified DIC score extends the use of a score to the Nordic countries.

Study I showed that antithrombin had a comparable discriminative ability with D-dimer and PT tests. The idea of replacing fibrinogen in the score with AT is tempting. However, its worldwide availability as a routine test is unclear. Recently, antithrombin was shown to correlate well with DIC score and to predict 28-day mortality.[285] Our study does not attempt to answer the question of whether AT behaves similarly in other patient groups outside ICUs or whether it improves the performance of the otherwise excellent score for overt DIC.

Surprisingly, diagnosis of DIC made by modified score did not independently predict 28-day mortality in the logistic regression model, although mortality rate increased with higher score. Other studies have quite congruently demonstrated higher mortality with higher scoring[286-288] and independent predictive power of DIC score.[60,64] The results from logistic regression analysis are highly dependent on the

imputed factors associated with the endpoint. The other studies have included remarkably fewer factors in the final analysis. In Study I, it seems rather obvious that when adjusted for disease severity (SOFA and APACHE II scores) the independent predictive power of dichotomous DIC diagnosis is eliminated. When the variables were chosen concordantly with other studies and dichotomous DIC diagnosis was replaced by the ordinal DIC score, modified score independently predicted 28-day mortality (unpublished data). This may indicate that DIC diagnosis encompasses an overly heterogeneous population of patients with coagulation disturbance, and the effect of DIC is emphasized only with a higher score, and thus, more severe disturbance.

6.3 TEM IN SEVERE SEPSIS

Study II demonstrated that patients with moderate coagulopathy in traditional coagulation tests but no overt DIC had no changes in TEM parameters, CT, CFT and α-angle relative to healthy controls. Only MCF was close to the upper normal limit of the reference range, but this trend did not reach statistical significance. DIC patients, however, had longer CFT, greater α-angle and lower MCF than both healthy controls and patients without DIC. Evidently, milder changes in global coagulation tests often seen in severe sepsis have no effect on whole-blood viscoelastic properties. In fact, these patients may be even prone to hypercoagulation once the process has sufficiently initiated. Only those with DIC exhibit signs of hypocoagulation. Prior to Study II, only a few groups had evaluated TEM/TEG in sepsis patients and none in DIC.[106,107,289] Since then, several larger studies have shown consistent results. Studies using ROTEM® in patients with severe sepsis or septic shock demonstrate normocoagulation within reference ranges[108,110,112,113] and hypofibrinolysis.[109,110,112] Interestingly, non-survivors and patients with higher SOFA score had reduced MCF and α-angle and prolonged CFT, suggesting hypocoagulation.[108,114] In addition, patients with overt DIC were hypocoagulable relative to those without.[110,113] All of these studies support the finding of Study II that the group of severe sepsis patients with normocoagulable TEM/TEG trace on average includes subgroups prone to hyper- and hypocoagulation. Hypocoagulable TEM/TEG, in turn, predicts mortality.[102,115,119]

The specific aim of Study II was to assess the applicability of TEM in the diagnosis of DIC. EXTEM MCF, CFT and α-angle discriminated ISTH score-defined DIC patients well, and the combination of MCF with either CFT or α-angle may further increase specificity. Only one other study has addressed this question with consistent results: Sharma et al.[118] studied a TEM score giving one point for each TEM parameter indicating hypocoagulation or hypofibrinolysis. If a patient received ≥2 TEM points, diagnosis of DIC could be set with a sensitivity of 95% and a specificity of 81%.

6.4 MMP-8 AND TIMP-1 IN SEVERE SEPSIS-ASSOCIATED DIC

Supported by several other studies, MMP-8 and TIMP-1 were strongly elevated in patients with severe sepsis as compared with healthy controls.[214,238,244,245,248-250] Both MMP-8 and TIMP-1 levels were highest on days 1 and 2, after which they slowly decreased. In patients with DIC, the changes over time in both MMP-8 and TIMP-1 levels were even more prominent than in patients without DIC. A few other studies have demonstrated essentially similar kinetics of MMP-8[238,247,250] and TIMP-1[249] in severe sepsis; however, none of them has separately studied patients with coagulation disturbance, and therefore, incidence of DIC in their material remains unclear.

Patients with DIC had higher TIMP-1 on days 1 and 2 and higher MMP-8 on day 2 than patients without DIC. Other clinical studies have not addressed this question in their study designs. Interestingly, particularly TIMP-1 had a profound correlation with both platelet count and several other coagulation tests, whereas MMP-8 correlated with only a severity of thrombocytopenia. Little is known about TIMP-1's independent interactions with coagulation, although similar correlations have been reported by two other studies.[244,251] In experimental studies, MMP-8 degraded TFPI[263,265] and fibrinogen,[266,290] and its effect on coagulation may be both pro- and anticoagulatory. In Study III, MMP-8 had no association with APACHE II or SOFA scores. Other studies have reported contradictory findings; whereas two larger studies reported an association with organ failure[238] and mortality,[238,248] two smaller studies found no association.[214,247] In small studies, like ours, random effect may confound the results.

In the last years, the independent role of TIMP-1 in organ dysfunction and prediction of outcome has aroused interest.[242,243,245,251,252,291] A variety of inflammatory factors can induce expression of TIMP-1,[292,293] and elevated levels are probably needed to assure efficient inhibition of up-regulated MMPs. Study I supported the understanding that TIMP-1 seems to carry more importance in severe sepsis than any MMP alone.

6.5 EXTRACELLULAR HISTONES AND HMGB1 IN SEVERE SEPSIS

The results of Study IV show that systemic nucleosomal hcDNA levels were higher in patients with platelet count <100 $x10^9$ /litre during the first five days in the ICU as compared with patients without thrombocytopenia. Admission hcDNA had a negative correlation with platelet count, and it predicted incidence of thrombocytopenia during the study period independently in logistic regression model. HcDNA level was stable for the first 48 hours, indicating that if elevated it remains high until the later phases of severe sepsis as well.

Especially free circulating histones have been linked to coagulation disorders in experimental and clinical studies.[170,188,189,193,206,207] Circulating histones may activate and aggregate platelets and induce macro- and microvascular thrombosis at least in

experimental circumstances.[185,189,294] Recently, a large study with 150 patients with deep venous thrombosis (DVT) and 195 controls demonstrated that elevated nucleosome levels with signs of neutrophil activation resulted in a 3-fold risk of DVT.[295]

Studies on nucleosomes interacting with coagulation in intensive care settings are scarce. Zeerleder et al.[208] could not confirm any correlation between nucleosome levels and coagulation in patients with disease severity ranging from fever to SIRS, severe sepsis and septic shock (n=69). However, a correlation became evident in a sub-group of patients with organ dysfunctions.[208] Unfortunately, the authors did not report precise data on correlation coefficients or scatterplots. In children with meningococcal sepsis (n=38), admission nucleosome levels had a significant correlation with DIC score and several coagulation parameters, including markers of enhanced fibrin formation. No data on thrombocytopenia *per se* were reported.[210] A small study with 20 patients with severe sepsis used the same hcDNA assay that we used in Study IV. HcDNA was reported to correlate with disease severity and coagulation parameters.[192] Very recently, hcDNA level was shown to be elevated in patients with overt DIC, to correlate with DIC score and to predict poor prognosis. Unselected critically ill patients presented only a minority of the study population (78/199), others having either solid or haematological malignancy, and among the patients with overt DIC (53/199) only 20 were critically ill. However, similar findings were achieved in both cancer and non-cancer patients.[194]

Although circulating nucleosomes seem to be associated with degree of coagulopathy, the contribution of nucleosomes to the development of thrombocytopenia in severe sepsis remains unclear. As relationships can be extremely multi-factorial in complicated illnesses, no straightforward interpretations can be drawn from causality based on correlation analyses. However, after assessing effects of other confounding factors, multivariable logistic regression model revealed an independent role of hcDNA in predicting thrombocytopenia.

In Study IV, hcDNA levels were associated with disease severity: patients with AKI of any stage had higher hcDNA level, as did those who died within 90 days of ICU admission. Previous studies have demonstrated consistent findings. A recent study aiming at validation of nucleosomes in prediction of new-onset sepsis and outcome showed that septic patients had higher levels of nucleosomes on admission than non-septic controls.[209] Admission level also correlated with APACHE II and maximal SOFA scores. Quite surprisingly, severity of sepsis did not have an influence on nucleosome levels,[179] unlike in a previous study by Zeerleder et al.[208] An experimental study suggested that CRP independently reduces histone toxicity against endothelium and histone-induced platelet aggregation.[205] The significance of this interesting finding in a clinical setting requires further investigations. Study IV confirms earlier findings that nucleosomes are associated with organ dysfunction and we extend this relationship to KDIGO-defined AKI.

Study IV demonstrated that also HMGB1, another nucleus-derived protein, is elevated in patients with thrombocytopenia, in AKI patients of any KDIGO stage and in 90-day non-survivors. HMGB1 had no value in predicting thrombocytopenia, but its significance increased in patients with more severe state of a disease. It independently predicted stage 3 AKI and 90-day mortality, which has been the case

in some other studies.[196,204] However, a previous large multi-centre cohort study on incidence and outcome of severe sepsis in Finland could not show any difference in HMGB1 levels between survivors and non-survivors.[296] In that study, the methodology of HMGB1 measurement was different from that in Study IV, serum was used instead of plasma, and outcome endpoint was hospital mortality instead of 90-day mortality. Diversity in the results of different studies questions the applicability of HMGB1 as a biomarker.

Nuclear proteins leaking from injured or apoptotic cells into extracellular space and circulation have been said to act as late markers of organ dysfunction and failure. In addition to being merely "markers", they may also cause and facilitate organ dysfunction.[186] Cytotoxicity is a consequence of interactions between highly cationic histones with charged parts of different cell membranes and the endothelium.[297] Thus, deleterious effects of extracellular histones only partly result from their interactions with coagulation. Moreover, histopathological findings are conflicting regarding the importance of fibrin depositions and thrombosis in the development of organ dysfunction.[140,141,147,298] Post-mortem human studies can be confounded by severe pre-mortem hypotension and ischaemia, as well as decelerated circulation of the organs potentially affecting the coagulation process. Based on Study IV, further conclusions about the effect of histones on different organs cannot be made, as we were unable to measure local concentrations of nuclear proteins.

6.6 STRENGTHS AND LIMITATIONS OF THE STUDY

Some strength and limitations of Studies I-IV should be addressed. First of all, these studies provide new information on the incidence of DIC, the applicability of diagnostic tools and the complicated pathophysiological process of severe sepsis and sepsis-associated coagulation disturbance.

At the time of publication, Study I was the inaugural study assessing the newly established score for overt DIC in a cohort of unselected ICU patients. The study was retrospective and based on clinical and laboratory data obtained from databases. Thus, no clinical diagnosis of DIC was available that could have served as the gold standard. However, the results are very consistent with other later studies, except for the role of DIC diagnosis in prediction of mortality. As discussed earlier, the statistical method may have influenced the results. Local modification of ISTH score for overt DIC can be regarded as a strength instead of a weakness. By replacement of PT with PT ratio, also other Nordic countries widely using that score can rely on the results.

Study II was designed as a response to a search for better tools to diagnose a degree of sepsis-related coagulation disturbance. However, we were not able to compare the results with any specific thrombin activation test. Also the study population was too small to allow any interpretations about the clinical significance of TEM results in predicting bleeding or thrombotic complications in an ICU setting.

Patients with severe sepsis may present different phases and severity of a disease even at ICU admission. In general, ICU patients, even those with more specifically

defined conditions, present a wide spectrum of diseases, chronic illnesses, prior medications and so forth. In critical illness research, one must accept this contingency and interpret the results while bearing in mind the heterogeneity. In smaller studies, heterogeneity of the patients may distort the results, especially if the kinetics of the investigated markers is unknown. Study II included only patients with severe sepsis at admission, Study III at admission or within 48 hours prior to admission and Study IV at admission or during the first 24 hours in the ICU. In the TEM study (II), we reported only admission samples. Kinetic study of MMP-8 and TIMP-1 (III) proved that the concentrations were highest at admission and on day 2, after which they slowly decreased; kinetics of both hcDNA and HMGB1 were rather stable for the first 48 hours. These findings reduce the possible effect of disease phase at the beginning of ICU admission.

Because of the preliminary and descriptive nature of Studies II-IV, expected levels of MMP-8, TIMP-1 and hcDNA between study groups were unknown, and thus, proper *a priori* power analyses and sample size calculations could not be performed. In Study II, we made *post hoc* calculations for differences of means with confidence intervals to assess the possible effect of B-type error. Despite small sample sizes and wide range of the results, we obtained fairly large differences between the medians in DIC and non-DIC patients (1.8-2.9-fold in MMP-8 and TIMP-1 concentrations). Naturally, these results have to be confirmed in larger studies.

6.7 METHODOLOGICAL CONSIDERATIONS

Regarding Study I, the issues concerning laboratory techniques and generalization of the results are discussed in Section 6.2.

Preanalytical factors may affect coagulation test results markedly.[299] In critically ill patients, blood samples for both clinical and scientific purposes are almost uniformly obtained from arterial cannula (Studies I-IV as well), whereas control samples are drawn often by venepuncture. A few studies have compared coagulation test results of simultaneous samples from arterial and venous lines and found no clinical differences. PT, platelet count and fibrinogen tests gave equal results in venous and arterial blood.[300,301] In venous blood, D-dimer was slightly elevated (0.03 mg/l) and AT decreased (2%). Although the results differed statistically, the finding had no clinical significance.[301] Comparison of TEM/TEG parameters showed that no clinically significant difference existed between arterial and venous samples.[301,302]

Automation and better standardization of TEM have overcome many earlier concerns regarding reliability. Still, however, ICU or operation room personnel instead of a hospital laboratory perform the tests, which may reduce the reproducibility. Also many patient-related factors may affect the results. In Study II, we excluded all those who had received prior anticoagulant therapy within one week or those with active bleeding. Due to partly different methodologies and activating reagents in TEM and TEG, comparability of the results is questionable.[303,304] Briefly, a panel of four TEM analyses seems to distinguish different types of coagulopathies better that kaolin-induced TEG-analysis alone.[305] This must be kept in mind also when assessing sepsis studies with either TEM or TEG and divergent findings.

In Study III, a central research laboratory with extensive knowledge and experience with the methods in question performed the MMP-8 and TIMP-1 analyses. Measurements were made from serum samples, which have been criticized for yielding inappropriately high concentrations of MMPs and TIMPs.[306] No other studies on MMP-8 and TIMP-1 in patients with DIC exist; however, two large studies on severe sepsis patients have confirmed the acquired TIMP-1 level.[244,251] Study III compares MMP-8 and TIMP-1 levels within a single study, avoiding generalizations. A larger study with more stable and comparable citrated plasma samples is needed.

In Study IV, histones were measured as hcDNA, giving a relative level of both mono- and oligonucleosomal fragments. Other studies using the same assay have reported either relative values comparable or higher than ours depending on study population[190,209,276] or have divided their data into "low" vs. "high hcDNA" sub-populations for further analyses.[191,192] So far, the method lacks clinical validation, and no reference range for an "acceptable" level of circulating nucleosomes exists. This method does not distinguish the origin of nucleosomes or give information about their biological activity.

In Study IV, HMGB1 was analysed from plasma samples that give essentially lower median values than the corresponding serum samples according to a recent methodological study (mean recovery of 27%, with a range of 14-32%).[307] HMGB1 stability after centrifugation was, however, good, and repeated freeze-thaw cycles did not affect the HMGB1 concentration. Our samples were centrifuged immediately after obtaining the sample, frozen to -70°C and thawed only for the final analysis.

6.8 ETHICAL CONSIDERATIONS

All studies were performed according to the Declaration of Helsinki, and the Helsinki University Hospital Ethics Committee of the Department of Surgery approved the study protocols. In Study I, need for an informed consent was waived due to the retrospective nature of the study. In Studies II-IV, patients or their next-of-kin gave written informed consents; in Study IV also a delayed consent was acceptable. None of the study designs caused disproportionate inconvenience to the patients, and blood sample volumes obtained were clinically insignificant to patients' health. The study protocols did not affect routine patient care in any way despite supplementary blood sampling.

6.9 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Research in the field of coagulation disorders in severe sepsis has come to the end of an era. Recent years have clearly demonstrated that coagulation is not a separate 'island' among the other systems, and a close interaction between inflammatory mechanisms, overall homeostasis of the cells and coagulation predominate during all phases of severe infection. More interest is focused on severe sepsis-associated loss of cellular homeostasis causing an inability to maintain normal functions and cell-cell interactions, which may lead to organ failure.[308] Although the details of

cellular dysfunction are beyond the scope of this thesis, several confluences with coagulation disturbance should be mentioned.

Taking into account the multifactorial nature and extreme complexity of severe sepsis, it is hardly surprising that large multi-centre trials investigating the usability and treatment effect of recombinant anticoagulants AT, aPC and TFPI failed to show any benefit in reducing mortality in a heterogeneous patient groups with severe sepsis syndrome.[309,22,160] Interestingly, later subgroup analyses have demonstrated that in more precisely defined subgroups, mostly those with disturbed coagulation and high risk of mortality, the treatments may have proven efficient.[58,59] According to a recent consensus recommendation of ISTH, none of the treatments despite routine thromboprophylaxis have been strongly recommended.[89] A large phase III trial on the effect of recombinant human soluble thrombomodulin in patients with severe sepsis-related DIC is ongoing. Also, promising results from a meta-analysis combining the data from all clinical studies on thrombomodulin encourage the continued development of treatments that simultaneously target both coagulation and inflammation.[310,311]

Despite the lack of globally accepted and registered treatment for DIC at the moment, it is, however, even more important to learn to recognize patients with developing coagulation disorders early, so that these patients can be selected for future studies from a large, heterogeneous population of severe sepsis patients. Although development of multiple organ failure, with a great certainty, is multifactorial in origin, coagulation is shown to be one strong contributor. Therefore, one could speculate that those patients with developing organ dysfunction with coagulation disorder may benefit from new 'tailor-made' therapies most.

Use of any score (ISTH, JMHW, JAAM)[7,87,88] for diagnosing DIC is an obvious improvement as compared with older times, when DIC was defined purely on the basis of well-known alterations in coagulation tests and clinical picture. In the future, however, the scientific community should pursue one globally accepted score for DIC instead of several local scoring systems. This study showed that ISTH score for overt DIC with local modification found those patients with higher morbidity and 28-day mortality. Fibrinogen had no value in DIC diagnosis in our patients, although in some other patient group it may have had more importance. Implication of AT either in the score or as a separate indicator of DIC is challenging, as its availability may be limited.

Since the original suggestion in 2001, Scientific and Standardization Committee has strongly encouraged the evaluation of new diagnostic tools, preferably based on easily accessible point-of-care methods. This study showed that TEM has a potential in the early and definite diagnosis of overt DIC. However, before introducing TEM in clinical practice in assessing degree of coagulopathy in patients with severe sepsis, several shortcomings must be addressed. Most importantly, results from two commercial assays using different methodologies and reagents need standardization. Second, instead of local adaptations, widely accepted and unified definitions of hypo- and hypercoagulation are needed. Third, as the methodology requires trained and committed personnel, it must be decided whether TEM will be integrated as part of the haematological laboratory or whether it will remain a pure point-of-care method close to the patient. The TEM/TEG traces also

require validation regarding critically ill patients' risk of bleeding and thrombosis. An intriguing perspective is that one could use TEM as a tool for assessing the need for transfusions and/or more efficient antithrombotic prophylaxis patient-wise. Instead of being an all-encompassing method for predicting outcome of large heterogeneous patient groups, TEM should be studied is better-restricted sub-groups, in which its advantages could be better achievable.

Neither traditional coagulation assays nor TEM/TEG respond sufficiently to the question of whether platelets function normally. For example, impaired platelet function measured by impedance aggregometry is associated with poor prognosis in severe sepsis.[312] Platelet function in disseminated intravascular coagulation requires further research, as only a few studies have addressed this question.[110]

This study provided new data on MMP-8 and TIMP-1 levels in severe sepsis-associated DIC. Whether these changes are dependent on only disease severity or inflammatory factors or whether they have a real role in coagulation remain to be investigated in larger studies. Especially TIMP-1 is interesting. It seems to have an independent role in inflammatory states, however it is unknown whether these changes are compensatory or detrimental *per se*. Unpublished findings on the power of TIMP-1 in the diagnosis of DIC demand further studies. Also, due to uncertainty of comparability of plasma and serum-based studies, these findings concerning a role of TIMP-1 in DIC must be confirmed and validated with plasma samples in other patient populations and geographical areas.

Pathophysiology of the development of organ dysfunction and failure in severe sepsis is at least as challenging as the multiple cellular interactions in severe sepsis, in general. This study provided new information on circulating nuclear proteins and their association with thrombocytopenia and organ dysfunction, with special reference to AKI defined by new KDIGO criteria. Release of nuclear proteins has been suggested as a major contributor of organ dysfunction and failure in sepsis, although these findings are mainly based on experimental data. In humans, data on local effects of nuclear proteins on development of organ dysfunction are lacking. Targeting both histones and HMGB1 with inhibitors in animal studies has proved very effective in preventing organ dysfunction.[313-317] These findings provide interesting future perspectives in developing new strategies in treatment of severe sepsis in humans. Especially being 'late-mediators' widens the time frame for treatment from the earliest moments of the disease to the later phases.

Critically ill patients form a heterogeneous group with several disease entities, different backgrounds and prior health states. Unique features of different subgroups of ICU patients prone to coagulation disturbances warrant further investigations. One very interesting subgroup with high frequency of coagulation abnormalities, often quite profuse need for blood products, severely disturbed permeability, and organ dysfunction, is patients with severe burn injuries. In these patients, interactions between coagulation and organ dysfunction are particularly poorly known.

In conclusion, DIC is an ultimate end-point of disturbed coagulation in the critically ill. Tools for earlier recognition of the patients with potential to develop severe

coagulation disorder are needed. The diagnostic repertoire of DIC can be significantly extended, as several recently studied markers have been properly validated.

7. CONCLUSIONS

The following conclusions can be drawn on the basis of this study:

1. The incidence of overt DIC is 31% in unselected ICU patients, as assessed by modified score for overt DIC. Of the components in the score, platelets discriminated patients with DIC excellently and PT ratio and D-dimer well, while fibrinogen proved useless. AT possesses comparable discriminative power as PT and D-dimer. Diagnosis of DIC based on modified score was not an independent predictor of 28-day mortality.

2. ROTEM® analysis revealed that severe sepsis patients with DIC were hypocoagulable, whereas those without DIC were prone to mild hypercoagulation. Traditional coagulation assays, simultaneously, demonstrated worsening coagulopathy. EXTEM MCF, CFT and α-angle discriminated patients with DIC well.

3. MMP-8 and TIMP-1 were strongly elevated in patients with severe sepsis. In DIC, TIMP-1 was higher on days 1 and 2, and MMP-8 on day 2, as compared with those sepsis patients without DIC. Especially TIMP-1 had a good correlation with coagulation tests and disease severity scores.

4. Systemic hcDNA and HMGB1 levels were elevated in patients with thrombocytopenia, in AKI patients of any KDIGO stage and in patients who died within 90 days. HcDNA correlated with platelet count and disease severity scores and was an independent predictor of thrombocytopenia. HMGB1 independently predicted AKI stage 3 and 90-day mortality.

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Since the beginning of this project, the common conception of pathophysiology of sepsis-related coagulation disturbances and the attempts to treat them have confronted several upheavals. Amidst these changes, it has sometimes been challenging to keep the focus of the project clear. I have learned that even in the middle of the hectic years at work and at home one must find the time to *read* a lot to gain a deeper understanding of the big picture. Today, I am grateful that I did not choose the straightest route, but spent some years wandering. It best serves my ultimate aim to become an analytic intensive care clinician.

I am deeply grateful to my supervisors for guiding me through this project: Professor Ville Pettilä has provided invaluable support, and I admire his vast experience in both research and clinical issues. His specific talent is to persuade others to push their limits. I am, however, convinced that his days contain 48 hours as opposed to the normal 24. Over the years, he has opened several Gordian knots and has patiently led me from a dead-end to the beginning of a new road again and again, for which I am most thankful. Docent Anne Kuitunen has provided unfailing encouragement and helped me profusely. Her extensive experience in coagulation has been indispensable. I know that many younger colleagues including myself have 'grown up' regarding her as the gold standard.

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